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BC 261-A1 A46

From: Goldberg, Jeanine
Sent: Friday, July 26, 2002 12:09 PM
To: STIC-ILL
Subject: please pull vdr poly breast cancer

1. Breast Cancer Research and Treatment (2002), 74(1), 1-7
CODEN: BCTRD6; ISSN: 0167-6806

2. Proceedings of the American Association for Cancer Research Annual Meeting, (March, 2000) No. 41, pp. 129.
Meeting Info.: 91st Annual Meeting of the American Association for Cancer Research. San Francisco, California, USA April 01-05, 2000
ISSN: 0197-016X.

3. ONCOLOGY RESEARCH, (1998) 10 (1) 43-6.
Journal code: 9208097. ISSN: 0965-0407.

4.INTERNATIONAL JOURNAL OF CANCER, (1999 Dec 10) 83 (6) 723-6.
Journal code: 0042124. ISSN: 0020-7136.

5. CANCER CAUSES AND CONTROL, (2000 Jan) 11 (1) 25-30.
Journal code: 9100846. ISSN: 0957-5243.

6. BRITISH JOURNAL OF CANCER, (2001 Jul 20) 85 (2) 171-5.
Journal code: 0370635. ISSN: 0007-0920.

7.CARCINOGENESIS, (1999 Nov) 20 (11) 2131-5.
Journal code: 8008055. ISSN: 0143-3334.

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to 4 μM BPDE in vitro, the lymphocytes were harvested for cytogenetic study. The average of simple chromatid breaks per cell (b/c) from a total of 50 metaphases per subject was used for statistical comparisons. Overall, cases had a greater mean b/c value (mean±SD, 0.53±0.22) than controls did (0.41±0.16). The difference was statistically significant (P<0.001). Using the control median b/c as the cut-off value for high and low sensitivity, high sensitivity was associated with a four-fold increased risk (Odds ratio, 4.00; 95% confidence interval, 1.61-9.97; adjusted for age and ethnicity). This preliminary finding suggests that increased sensitivity to tobacco carcinogens may play a role in the etiology of breast cancer. (Supported in part by HIH grant CA70264, CA55769, and CA70334).

#820 *WAF-1 (P21)* AND *P53* POLYMORPHISMS IN BREAST CANCER. Channa K C Keshava, B. L Frye, M. S Wolff, and A. Weston, *Mount Sinai Med Ctr, New York, NY, and Niosh,, CDC, Morgantown, WV*

Previous studies have indicated that certain *p53* polymorphisms confer an increased risk of breast cancer (ORs and 95%CIS = 2.9, 1.4 – 6.3 *Carcinogenesis* 17: 1313, 1996; 2.5, 1.3 – 4.8 *Cancer Epidermiology, Biomarkers and Prevention* 6:105, 1997; 1.5, 1.1 – 2.0, *Anticancer Research* 18: 2095, 1998). *p53* is a transcription factor for *Waf-1/p21* a cyclin-dependent kinase inhibitor, which is also polymorphic. To test the hypothesis that minor variants (F = 0.10 Caucasians, 0.27 Latinas, 0.34 African Americans) of a codon 31 polymorphism of *Waf-1* are involved in this process, genotypes were determined by PCR/RFLP for 355 women (122 cases and 233 controls) enrolled in a breast cancer case-control study. No increased breast cancer risk was associated with inheritance of minor variants of *Waf-1* (OR = 1.1, 95%CI = 0.7 – 1.6). Similarly, analysis by both race and menopausal status was unable to find any association. Finally, despite an increased risk for Caucasians associated with the *p53* genotype (*CEBP* 1997), no risk was found to be associated with *Waf-1* alleles independently or in combination with *p53* alleles (OR = 1.1, 95%CI = 0.3 – 4.7).

#821 CHARACTERISTICS OF P53, HER/*NEU* AND *BCL-2* IN A LOW RISK BREAST CANCER POPULATION OF CHINESE PATIENTS FROM MAINLAND CHINA. XiaoTan Qiao, Karen S Fiderici, Zeng Si, ChangBan Gong, GongHa Zhou, Yan Li, Lin Wang, KeFeng Dou, Kenneth S van Golen, Sofia D Merajver, and Charles D Mackenzie, *BenXi Gen Hosp, BenXi, People's Rep of China, China-Japan Friendship Hosp, BeiJing, People's Rep of China, Michigan State Univ, East Lansing, MI, Univ of Michigan, Ann Arbor, and XiJing Hosp, Xian, People's Rep of China*

Reliable epidemiological data reveal striking differences in breast cancer risk between the North American Caucasian and Chinese Asian populations. We hypothesize that these differences in risk reflect in part, different pathways of breast carcinogenesis, which may, in turn be due to epigenetic or environmental variables. To begin to test this hypothesis, we investigated a cohort of 178 patients breast cancer samples from mainland China. The tumors were analyzed for descriptional parameters such as age, stage, ER/PR status, and grade as well as molecular genetic alterations in p53, HER-2/*neu*, and Bcl-2. For p53, HER2/*neu* (*c-erbB-2*), and Bcl-2, 14.2%, 23.1% and 66.4% stained positively by immunohistochemistry. HER2/*neu* gene amplification was detected by differential polymerase chain reaction methods and 29.1% of specimens were positive. Sixty-four samples were evaluated for p53 gene point mutations in exon 5 to 9 by PCR-single strand conformation polymorphism assay, followed by gene sequence analysis: only 1/64 (1.56%) was found to be positive for a missense transition mutation at codon 151, a CpG site. The results demonstrated that the Western (high breast cancer risk group) and Chinese (low risk group) populations have similar phenotypic features and also similar proportions of genetic alterations in these 3 key molecular markers.

#822 BREAST CANCER INCIDENCE AMONG A COHORT OF WOMEN WITH BENIGN BREAST DISEASE. Angela C Blount, Usha Raju, Judith Abrams, Michelle Jankowski, S David Nathanson, Sandra R Wolman, Maria J Worsham, and Christine C Johnson, *Henry Ford Health System, Detroit, MI, Uniformed Service Univ of the Health Sci, Bethesda, MD, and Wayne State Univ, Detroit, MI*

The risk of developing breast cancer has been reported to be increased among women with a history of benign breast disease (BBD). A cohort of women diagnosed with BBD from 1981-1994 was established to investigate this relationship in a large health care system. Women were eligible for entry with an initial index BBD biopsy performed during this time period. A diagnosis of breast cancer prior, concurrent or within 6 months of the index BBD biopsy ruled women ineligible for the cohort. The archived pathology reports of all breast biopsies were retrieved and reviewed by an expert breast pathologist to identify specimens containing only BBD lesions. The slides were microscopically reviewed for confirmation of the diagnosis utilizing a universal diagnostic terminology system. All cohort members were followed from their index BBD biopsy for the subsequent occurrence of breast cancer. During cohort establishment, 5254 women were found to be eligible and 116 ineligible. Slide review revealed the lesions were primarily proliferative (65%), with 30% non-proliferative, and 4% atypical ductal or lobular hyperplastic. The cohort yielded 167 cases of breast cancer detected through July 1999. With 48,201 person-years of follow-up, the average incidence rate was 346.5 per 100,000 (95% confidence interval [CI], 295.9-400.8), ranging from 298.3 (95% CI, 148.9-534.0) in the 1981 cohort year to 530.8 in 1994 (95% CI, 254.8-976.6). In comparison to 1991-1995 SEER rates of 353.8 nationally and 363.6 per 100,000 for the metropolitan Detroit area among women aged 50 and older, breast cancer incidence in this BBD cohort does not appear to differ from the general population.

#823 EVALUATION OF PROPHYLACTIC OPTIONS FOR ASHKENAZI JEWISH WOMEN WITH A BRCA MUTATION: A DECISION ANALYSIS. Lesley-Ann Natasha Miller, and Mendel E Singer, *Case Western Reserve Univ Sch of Medicine, Cleveland, OH*

Ashkenazi Jewish women have a high prevalence (about 2.5%) of three specific BRCA1/2 mutations that are associated with an increased risk of developing breast or ovarian cancer. The authors developed a Markov decision model and used Monte Carlo simulation to evaluate the implications of various prophylactic options for a 40 year old woman who tests positive for any one of these mutations. Prophylactic options considered included prophylactic mastectomy (PM), prophylactic oophorectomy (PO), both PM and PO, tamoxifen chemoprevention, and increased screening. Parameter estimates were taken from SEER cancer statistics and the published literature. Outcomes considered were additional life expectancy and quality-adjusted life years (QALYs). We assumed that PO would reduce the risk of ovarian cancer (OC) by 46% and breast cancer (BC) by 25%, PM would reduce the risk of BC by 90%, and tamoxifen would reduce the risk of BC by 44%. Increased screening was defined as biennial mammography and clinical breast exam. We postulated that this increased screening would lead to beneficial gains associated with an earlier stage of diagnosis. The results indicate that the strategy of both PM and PO offered the greatest benefit in terms of increased life expectancy. However, after adjusting for quality of life (QOL), increased screening becomes the preferred strategy. For all surgical or chemopreventive strategies, the loss in QOL more than offset the benefit of the associated risk reduction. Time discounting of future life years had no impact on the results. QOL considerations may have a profound impact on choosing the optimal BC/OC prophylaxis.

#824 ASSOCIATION BETWEEN BREAST CANCER AND THE THREE DIFFERENT VITAMIN D RECEPTOR GENE POLYMORPHISMS TAQI, BSMI AND APAI. Diana Lueftner, M. Schweigert, K. Engellandt, P. Petrides, I. Roots, K. Possinger, and I. Cascorbi, *Humboldt Univ Berlin, Berlin, Germany*

Breast cancer (BRCA) growth is influenced by vitamin D. We investigated the distribution of the *TaqI* (T/t), *BsmI* (B/b) and *ApaI* (A/a) VDR gene polymorphisms in 247 BRCA patients and 248 age-matched controls. After DNA extraction from white blood cells, VDR genotypes were determined by polymerase chain reaction (PCR) amplification followed by restriction enzyme digestion of the PCR product. The mean age for BRCA patients (and controls) was 60.4 (60.1) years with a range from 31–90 (31–91) years. The VDR genotype distribution for BRCA patients (in comparison to controls) was as follows: BB: 17.8% (17.3%); Bb: 46.6% (48%); bb: 35.6% (34.7%); AA: 26.7% (26.6%); Aa: 49.8% (53.6%); aa: 23.5% (19.8%); TT: 37.7% (39.1%); Tt: 47.4% (51.6%); tt: 15.0% (9.3%). The VDR genotype distribution was statistically not different between BRCA patients and controls for the *BsmI* and *ApaI* genotypes. However, for *TaqI* an increase of the genotypes TT + Tt vs. tt could be found (odds ratio: 1.72; CI: 0.99–2.99, p=0.052). Combined analysis adjusted for age and considering all genotypes revealed a relative risk of TT vs. tt of 3.02 (CI: 1.19-7.71, p=0.02) to develop breast cancer. This finding is important for the screening of risk families and for replacement therapy in hospitalized patients who generally show a decreased vitamin D level.

CELL AND TUMOR BIOLOGY 6: Proteases I

#825 RAPID TRAFFICKING OF MT1-MMP TO THE CANCER CELL SURFACE FROM A POST-GOLGI STORAGE POOL RESULTS IN EXPLOSIVE CELL SURFACE ACTIVATION OF LATENT MMP-2. Stanley Zucker, Michelle H Hymowitz, Cathleen E Conner, and Jian Cao, *SUNY - Stony Brook, Stony Brook, NY, and VA Med Ctr, Northport, NY*

Pericellular matrix degradation during cancer invasion is dependent on activation of proMMP-2 by Membrane Type 1-Matrix Metalloproteinase (MT1-MMP). We herein report that concanavalin A (con A) or phorbol (PMA) treatment of HT-1080 fibrosarcoma cells is followed by MT1-MMP induced activation of proMMP-2 on the cell surface within 30 min. Surface biotinylation, immunoprecipitation, and ^{125}I-TIMP-2 binding techniques were employed to characterize MT1-MMP appearance on the cell surface. Con A-induced trafficking of MT1-MMP from a post-Golgi compartment (endosomal/secretory) to the cell surface occurred within 10 min. Rapid MT1-MMP trafficking was accelerated by brefeldin A, a Golgi inhibitor and chloroquine, a lysosome inhibitor; cycloheximide, a protein synthesis inhibitor, had minimal early effect. Rechallenge of HT-1080 cells with con A 3 hr later demonstrated a requirement for new protein synthesis and transit through the Golgi (inhibited by cycloheximide/brefeldin A). Con A enhancement of MT1-MMP mRNA synthesis was not noted before 18 hr. After binding to cell surface MT1-MMP, ^{125}I-TIMP-2 is internalized and secreted as an intact protein after 3 hr. These results are consistent with an intracellular recycled storage pool for MT1-MMP which is readily available to invasive cancer cells.

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6134 56

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Oncology Research, Vol. 10, pp. 43–46, 1998
Printed in the USA.

0965-0407/98 $10.00 + .00

Vitamin D Receptor Gene Polymorphism Is Associated With Metastatic Breast Cancer

Marco Ruggiero,*[1] Stefania Pacini,* Stefano Aterini,* Carlo Fallai,† Carla Ruggiero,† and Paolo Pacini†

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(Submitted September 23, 1997; sent for revision November 7; received and accepted December 5, 1997)

The vitamin D receptor (VDR) has been detected in breast tumor cells. We tested the hypothesis that VDR gene polymorphism might influence the outcome of women affected by breast cancer. A total of 88 breast cancer patients were recruited: 50 women were affected by newly diagnosed breast cancer whereas 38 women suffered from relapsing disease. The individual genetic pattern for VDR was evaluated by DNA extraction followed by PCR amplification of the VDR gene, and digestion with the restriction enzyme *Bsm*I. In 167 healthy women, participating in the osteoporosis prevention trial and being used as a control, we detected 121 Bb heterozygotes (72%), 26 homozygotes for the bb alleles (16%), and 20 homozygotes for the BB alleles (12%). In the newly diagnosed breast cancer group the occurrence of Bb patients was 58% (29/50); bb patients represented 22% (11/50), and BB cases were 20% (10/50). The VDR frequency distribution in the control and primary disease patient groups was not statistically different. In the metastatic cancer group, the prevalence of the bb genotype (14/38; 37%) was double the percentage of control subjects, whereas the percentage of BB women with metastases was half the control group (2/38; 5%). Women who were homozygous bb appeared to have almost a four times higher risk of developing metastases than BB women. Whatever the molecular mechanisms underlying the VDR effects in cancer cells, we believe that the VDR gene polymorphism may represent an important determinant in the evaluation of women affected by breast cancer and might help design targeted therapy.

Key words: Breast cancer; Vitamin D receptor gene; Polymorphism; Disease susceptibility

Vitamin D_3, similarly to other steroid hormones, interacts with an intracellular receptor protein, the vitamin D receptor (VDR[2]), in order to initiate a broad spectrum of biologic responses. Apart from the classic control of calcium homeostasis, recent evidences support the involvement of vitamin D in the growth and differentiation of normal and malignant cells, suggesting a role for vitamin D in tumor therapy (1,2). In breast cancer cell lines, a biphasic response after vitamin D treatment has been observed, with lower doses stimulating proliferation and higher doses inhibiting cell growth (3). Moreover, the presence of VDR in breast tumor cells has been associated with a longer disease-free survival when compared with VDR-negative carcinomas (4).

Recently, common allelic variants in the gene encoding the VDR have been described, on the basis of the presence of the *Bsm*I endonuclease restriction site, labeled as "b," or of its absence, labeled as "B." At the molecular level, the inherited VDR gene polymorphism has been associated with differences in the transcriptional activity and mRNA stability (5). VDR genotypes have been related to different bone mineral density, suggesting a susceptibility to osteoporosis for women with the BB allele variants (5,6), and to the abnormal parathyroid function observed in primary as well as secondary hyperparathyroidism, where patients with the bb variants exhibit enhanced cell proliferation and higher parathyroid hormone (PTH) serum concentrations (7–9). Pathophysiological data indicate that the VDR gene alleles play differential actions at the site of the vitamin D circulating levels, parathyroid hormone suppression and intestinal calcium absorption, conveying the impression of a functional defect in the VDR expressed by the BB allelic variants (5,10–12).

Because physiological doses of vitamin D stimulate proliferation in breast cancer cells (3), we hypothesized that women with different VDR gene alleles affected by breast carcinoma would have distinct clinical course in the cancer development and/or relapse. Because the bb genotype of the VDR seems to be associated with a better receptor efficiency, we tested the hypothesis that women with such genotype might present a less favorable outcome, considering that cancer cells were more sensitive to the vitamin D proliferative action.

MATERIALS AND METHODS

A total of 88 consecutive breast cancer patients were recruited at the Oncology Radiotherapy Centre of the University of Firenze and were used as the breast cancer case group, in agreement with the previously reported sampling method for the study of prostate cancer and VDR (13). Fifty women were affected by newly diagnosed breast cancer whereas 38 women, who had undergone previous breast cancer surgery, suffered from relapsing disease at the time of the present survey. A total of 167 noncancer women, participating in the osteoporosis prevention trial, from the same geographic area and with the same ethnic characteristics in order to overcome the extreme ethnic and regional differences in the

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[2]Abbreviations used: VDR, vitamin D receptor; PTH, parathyroid hormone.

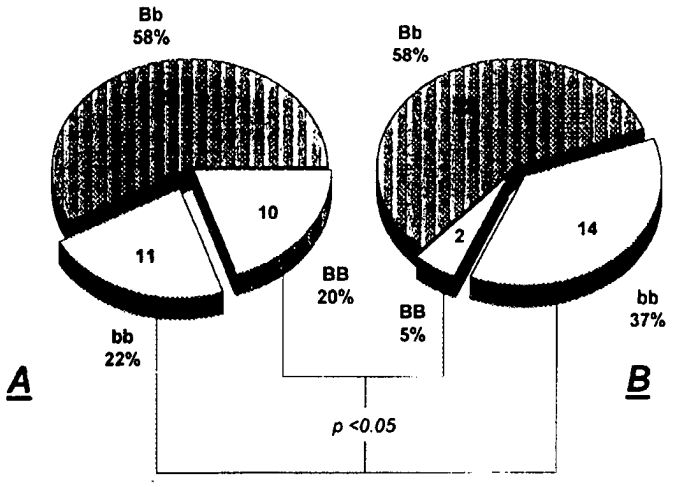

Figure 1. Distribution of VDR alleles in primary breast cancer (A) and in relapsed disease (B).

pattern of prevalence of the VDR alleles, were enrolled and used as a control. Blood samples were obtained from patients and controls and DNA was extracted.

DNA Extraction

Genomic DNA was extracted from blood using an inorganic method, based on proteinase K (Sigma Chemical Co., St. Louis, MO, USA) digestion without phenol. About 5 ml of blood was diluted with 35 ml of cold Tris 20 mM/EDTA 5 mM, then iced for 20 min and centrifuged at 3500 rpm for 15 min at low temperature (4°C). The pellet obtained was resuspended in a vortex in 4 or 5 ml of TE 20-5, then brought to a volume of 40 ml with the same solution. It was subjected to one or two of these lavages until it became white. The white pellet was resuspended by vortexing in half the initial volume of blood in TE 20-5. Then Sarcosyl (*N*-Lauroylsarcosine, sodium salt; Sigma Chemical Co.) was added to a final concentration of 1%, gently mixing by hand, and finally proteinase K was added in a final concentration of 200 µg/ml. The sample was incubated at 42°C overnight by gentle agitation. The day after, ammonium acetate was added to a final concentration of 3 M, mixing well. DNA was then precipitated with cold absolute ethanol twice and finally resuspended in Tris 20 mM/EDTA 1 mM.

Amplification and Digestion With Restriction Enzyme

PCR of the DNA sequence flanking the *Bsm*I site was used to facilitate genotyping of subjects with a MJ Research, Inc. (Watertown, MA, USA) thermal controller. Genomic DNA is amplified with TaqPolymerase (Polymed, Firenze, Italy) and gene-specific primers of 30 and 23 bp, during 32 cycles. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The reaction mixture contained: 1× PCR buffer [(670 mM Tris-HCl, pH 8.8, at 25°C; 160 mM $(NH_4)_2SO_4$; 0.1% Tween-20], $MgCl_2$ (2 mM), dNTP (200 µm of each), 9.6 ng/µl of each primer, and 2.0 units of PolyTaqDNA polymerase. Amplified DNA was analyzed on a 2% agarose gel: a single 870-bp band was obtained. Then 10 µl of the amplified DNA was restricted with *Bsm*I (3 U) in the appropriate reaction buffer (1×) provided by "Celbio" (Milan, Italy), according to the supplier's specification. Restricted DNA was still analyzed on a 2% agarose gel. Three different patterns of bands could be obtained: a single 870-bp band, corresponding to the amplified DNA without restriction; a 640- and a 230-bp band, indicating the presence of the restriction site for *Bsm*I; three bands of 870, 640, and 230 bp, indicating a heterozygote genotype.

Statistical Analysis

Association between disease and genotype was evaluated by estimating the chi-square statistics, the relative risk (RR), and 95% confidence intervals, using the SPSS statistical analysis system. In agreement with previous studies, if the VDR alleles have a significant effect on disease, it would be expected that homozygotes provide the clearest evidence of this difference. Therefore, heterozygotes are not shown in Table 1.

RESULTS

Among the 167 control women we detected 121 heterozygous Bb subjects (72%), 26 homozygous bb (16%), and 20 homozygous BB (12%). In the newly diagnosed breast cancer group the occurrence of heterozygous Bb patients was 58% (29/50); homozygous bb patients represented 22% (11/50), and homozygous BB cases were 20% (10/50) (Fig. 1). Among the women who presented metastases, 58% (22/38) were heterozygous Bb, 37% (14/38) were homozygous bb, and 5% (2/38) were homozygous BB ($P < 0.05$ vs. newly diagnosed cancer patients) (Fig. 1).

The VDR frequency distribution in the control and primary disease patient groups was not statistically different and no genotype was linked with the occurrence

Table 1. Distribution of VDR Homozygous Genotypes Among Breast Cancer Patients and Control

	bb (%)	BB (%)	*P* vs. Controls	Relative Risk bb vs. BB	95% CI
Controls	26 (16)	20 (12)			
Primary breast cancer	11 (22)	29 (20)	0.75	0.89	0.44–1.81
Metastatic breast cancer	14 (37)	2 (5)	0.025	3.85	0.96–15.41

Allelic variants in the gene encoding the VDR were evaluated by DNA extraction followed by PCR of the VDR gene, digestion with the restriction enzyme *BsmI*, and labeled as "b" on the basis of the presence of the *BsmI* endonuclease restriction site, or "B" in case of its absence. Homozygotes only are reported in order to provide the clearest evidence of any significant effect related to the VDR alleles. Refer to the Materials and Methods section for details.

of breast carcinoma (Table 1). In the metastatic cancer group, the prevalence of the bb genotype was double the percentage of control subjects with the same alleles, whereas the quota of BB women with metastases was half the control group, as a percentage (Table 1). The women who were homozygous bb appeared to have almost a four times higher risk of developing metastases than women who were homozygous BB (Table 1).

DISCUSSION

In the present study we investigated the association of the different VDR genotypes with the occurrence of breast cancer, primary as well as metastatic disease. Our observations convey the impression that genotype has no evident impact on the development of the primary tumor. On the other hand, as far as the occurrence of relapsing cancer is concerned, it appears that homozygous bb women have a higher risk of tumor recurrence.

The present data are quite in agreement with a recent report that has shown the association of prostate cancer with the VDR gene polymorphism (13). Men who expressed the tt homozygous genotype, which is the equivalent of the BB alleles, appeared to have one third the risk of developing prostate cancer in comparison to men with other VDR genotypes. The authors concluded that the higher serum levels of vitamin D associated with this genotype would decrease the risk for prostate cancer. Alternatively, the lower prevalence of metastatic disease in BB women and of primary prostate cancer in tt (BB) men could be related to the reduced sensitivity of the VDR associated with the BB genotype to the vitamin D action, as previously reported in both physiological (11,12,14) and pathological (5–9) conditions.

VDR have been detected in a variety of cell lines obtained from human cancers (1,15). High concentrations of vitamin D have been reported to inhibit the proliferation of tumor cells (16–19). Actually, in breast cancer, low, physiological levels of vitamin D play a proproliferative role in cell growth, in the presence of estradiol, whereas greater doses are antiproliferative (3). In this regard, our data do not allow to draw any definite conclusion on the role of VDR alleles in the etiology of breast cancer, because no difference between the three genotypes has emerged in women affected by primary disease, in comparison to control subjects; other genetic mutation (20,21) and polymorphism (22) might play a relevant role. On the other hand, the vast majority of women with metastases showed the b allele, either in the homo- or heterozygous form, whereas only 5% of the women with relapsing disease expressed the BB genotype. It is conceivable that, in the group of BB women, cancer cells, at least in the metastases microenvironment, would be less sensitive to the vitamin D proproliferative effects, unlike bb and Bb patients. One year later, only 4 out of the 50 newly diagnosed disease patients had developed recurrent disease, so that no definite assumption can be surmised. Prospective studies are requested to clarify this issue.

In conclusion, whatever the molecular mechanisms underlying the VDR effects in cancer cells, we believe that the VDR gene polymorphism represents an important determinant in the evaluation of women affected by breast cancer, which needs to be framed in the overall health picture of every single woman. The early identification of genotypically determined risk factors for cancer development or recurrence might help design targeted therapy.

ACKNOWLEDGMENTS: We are indebted to Dr. Laura Nicastro for helpful suggestions and discussions, and to Ms. Sophie Minns for assistance in editing and preparing the manuscript. This study was supported by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), and the Associazione Italiana per la Ricerca sul Cancro (AIRC).

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STIC-ILL RC261.A1 I57

From: Goldberg, Jeanine
Sent: Friday, July 26, 2002 12:09 PM
To: STIC-ILL
Subject: please pull vdr poly breast cancer

1. Breast Cancer Research and Treatment (2002), 74(1), 1-7
   CODEN: BCTRD6; ISSN: 0167-6806

2. Proceedings of the American Association for Cancer Research Annual Meeting, (March, 2000) No. 41, pp. 129.
   Meeting Info.: 91st Annual Meeting of the American Association for Cancer Research. San Francisco, California, USA April 01-05, 2000
   ISSN: 0197-016X.

3. ONCOLOGY RESEARCH, (1998) 10 (1) 43-6.
   Journal code: 9208097. ISSN: 0965-0407.

4. INTERNATIONAL JOURNAL OF CANCER, (1999 Dec 10) 83 (6) 723-6.
   Journal code: 0042124. ISSN: 0020-7136.

5. CANCER CAUSES AND CONTROL, (2000 Jan) 11 (1) 25-30.
   Journal code: 9100846. ISSN: 0957-5243.

6. BRITISH JOURNAL OF CANCER, (2001 Jul 20) 85 (2) 171-5.
   Journal code: 0370635. ISSN: 0007-0920.

7. CARCINOGENESIS, (1999 Nov) 20 (11) 2131-5.
   Journal code: 8008055. ISSN: 0143-3334.

THANK YOU

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Int. J. Cancer: **83,** 723–726 (1999)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

ASSOCIATION OF A VITAMIN D RECEPTOR POLYMORPHISM WITH SPORADIC BREAST CANCER DEVELOPMENT

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Breast cancer is the leading cause of cancer death among Australian women and its incidence is annually increasing. Genetic factors are involved in the complex etiology of breast cancer. The seco-steroid hormone, 1,25 dihydroxy vitamin D_3 can influence breast cancer cell growth *in vitro*. A number of studies have reported correlations between vitamin D receptor (VDR) gene polymorphisms and several diseases including prostate cancer and osteoporosis. In breast cancer, low vitamin D levels in serum are correlated with disease progression and bone metastases, a situation also noted in prostate cancer and suggesting the involvement of the VDR. In our study, 2 restriction fragment length polymorphisms (RFLP) in the 3′ region (detected by ApaI and TaqI) and an initiation codon variant in the 5′ end of the VDR gene (detected by FokI) were tested for association with breast cancer risk in 135 females with sporadic breast cancer and 110 cancer-free female controls. Allele frequencies of the 3′ ApaI polymorphism showed a significant association ($p = 0.016$; OR = 1.56, 95% CI = 1.09–2.24) while the *Taq*I RFLP showed a similar trend ($p = 0.053$; OR = 1.45, 95% CI = 1.00–2.00). Allele frequencies of the FokI polymorphism were not significantly different ($p = 0.97$; OR = 0.99, 95% CI = 0.69–1.43) in the study population. Our results suggest that specific alleles of the *VDR* gene located near the 3′ region may identify an increased risk for breast cancer and justify further investigation of the role of *VDR* in breast cancer. *Int. J. Cancer* 83:723–726, 1999.
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Breast cancer is the most common malignancy among the Australian female population with women having a 1 in 11 chance of developing the disease before the age of 75. Currently, breast cancer accounts for 18.6% of annual cancer deaths with the incidence increasing by 4% from 1995 to 1996 (Coates and Armstrong, 1997). Breast cancer, like other cancers, exists in both sporadic and inherited forms with the latter accounting for up to 10% of all breast cancer cases (Jones *et al.*, 1995). Familial breast cancer is commonly due to inheritance of germ line mutations, however sporadic breast cancer is often the result of a multifactorial etiology that includes a genetic component (Kelsey, 1993). A correlation between family history and increased risk of breast cancer development supports the hypothesis that genetic factors are involved in the etiology of sporadic breast cancer (Jones *et al.*, 1995). Complex hormonal influences are also important in the development of breast cancer as the incidence rises steeply in post-menopausal women (Devilee and Cornelisse, 1994). It is possible that the impact of particular genes in sporadic cancer may be modulated by the hormonal milieu.

Numerous studies have found that vitamin D and its analogues reduce cell proliferation in breast cancer cell lines and tumor samples and may hence play a protective role against breast cancer development (Jenkins *et al.*, 1997). In addition to the traditional role of vitamin D in controlling calcium balance, the active form of vitamin D [1,25$(OH)_2$D3] has potent anti-proliferative effects on breast, prostate and colon cancer cells in culture. This may be derived from stimulation of cellular differentiation or direct regulation of cell cycle genes such as *p53* and *p21* (James *et al.*, 1996). Polymorphisms in the receptor generate restriction sites within the gene that may potentially influence the stability of mRNA and vitamin D expression (Jenkins *et al.*, 1997; Ingles *et al.*, 1997*a*,*b*). Low serum levels of 1,25$(OH)_2$D3 have been reported to correlate with breast cancer disease progression and the development of bone metastases (Mawer *et al.*, 1997), hence polymorphisms in the vitamin D receptor (*VDR*) gene could be associated with breast cancer development.

VDR gene polymorphisms located at the 3′UTR have been found to be associated with prostate cancer, bone mineral density and osteoporosis (Ingles *et al.*, 1997*a*,*b*; Sainz *et al.*, 1997) while the 5′ polymorphism is involved in peak bone density (Harris *et al.*, 1997). In this study, 3 sequence polymorphisms, 1 from the 5′ region and 2 from the 3′ region of the *VDR* gene, were analyzed for involvement in sporadic breast cancer development in an Australian breast cancer affected population.

MATERIAL AND METHODS

Subjects

The affected tested population comprised 135 females previously diagnosed with breast cancer. Each was without a known family history of breast cancer, although there may have been unidentified familial cases (not expected to exceed 5%). The control population comprised 110 females with no history (either familial or personal) of cancer of any type. The mean ages of the study groups were 59.6 ± 1.01 years for the cases and 49.7 ± 1.35 years for the controls. The median age of the affected population was 60 years with a range of 31–88 years; and for the control population, the median age was 49 years with a range of 20–81 years. The affected population was recruited from 416 breast cancer affected individuals in collaboration with the Pathology Department, Gold Coast Hospital, Southport, and the control population through the Genomics Research Centre, Griffith University, Southport, Queensland, Australia. Control individuals were recruited over a 4-year period and at the time of donation, completed a detailed questionnaire outlining personal and family history of a variety of disorders. Only individuals with no family history of cancer or precancerous conditions were selected for involvement in the study. All individuals were informed of the objectives of the study and consent was received for participation. Blood samples were then collected from participating individuals and DNA extracted by standard procedures (Lea *et al.*, 1998).

Genotyping

Three polymorphic restriction fragment length polymorphisms (RFLPs) were genotyped; the FokI polymorphism in the initiation codon at the 5′ end and 2 polymorphisms (*Apa*I and *Taq*I) in the 3′ region of *VDR*. These polymorphic regions were amplified by standard, unlabeled oligos (Table I) followed by restriction enzyme digestion corresponding to each RFLP.

For detection of the initiation codon polymorphism, 50–100 ng genomic DNA was amplified with 1 × polymerase chain reaction

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Received 6 February 1999; Revised 23 June 1999

TABLE I – OLIGO SEQUENCES

Primer	Nucleotide sequence
*Apa*I and *Taq*I RFLP	
Forward	CAGAGCATGGACAGGGAGCAAG
Reverse	GCAACTCCTCATGGGCTGAGGTCTCA
*Fok*I RFLP	
Forward	GATGCCAGCTGGCCCTGGCACTG
Reverse	ATGGAAACACCTTGCTTCTTCTCCCTC

(PCR) buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each primer and Taq polymerase in a 20-μL final volume on a Corbett (Sydney, Australia) PC-960 thermocycler. Cycles consisted of a 4-min denaturation at 94°C followed by 30 cycles of 94°C for 1 min and 60°C for 1 min then a final extension at 60°C for 7 min. PCR products were digested with FokI (1 U at 37°C) and electrophoresed on 2% ethidium stained agarose gels. Genotypes were denoted as FF (272 bp), Ff (272, 198, 74 bp) or ff (198, 74 bp).

For detection of the *Apa*I and *Taq*I RFLPs, amplification required 50–100 ng genomic DNA with PCR Premix Optimisation Buffer E (Epicentre Technologies, Madison, WI), 0.2 μM of each primer and Taq polymerase in a 25-μL reaction volume. Amplification was then performed on a Perkin Elmer (Foster City, CA) thermocycler with a 94°C initial denaturation for 4 min followed by 5 cycles of 94°C for 45 sec, 64°C for 60 sec and 72°C for 2 min; and a further 25 cycles of 94°C for 30 sec, 64°C for 30 sec and 72°C for 45 sec. Following amplification, PCR products were digested with ApaI (2 U at 37°C) or TaqI (2 U at 65°C) and electrophoresed on 2% agarose gels stained with ethidium bromide. Genotypes were determined as AA (740 bp), Aa (740, 515, 225 bp) or aa (515, 225 bp) for *Apa*I polymorphism and TT (490, 245 bp), Tt (490, 290, 245, 205 bp) or tt (290, 245, 205 bp) for *Taq*I polymorphism (dominant alleles denoting absence of restriction site).

Statistical analyses

To determine significance of polymorphisms in the case compared with the control population, allele frequencies for each RFLP were compared statistically by χ^2 analysis in contingency tables and odds ratios (OR). The SPSS 7.5 statistical package was used for this analysis. Statistics were calculated using 95% confidence intervals (CI; $p < 0.05$ statistically significant). Simple factorial analysis of variance (ANOVA) and logistic regression were used to test for the influence of covariates on gene effects.

RESULTS

We have examined 3 restriction polymorphisms of the *VDR* gene in a sporadic breast cancer affected and a suitable control population. The frequencies of each allele for the populations are shown in Table II. As can be seen in Table II, when the allele frequencies of the *Apa*I polymorphism were compared between the affected and control populations, a significant difference was observed ($\chi^2 = 5.83$, df = 1, $p = 0.016$). The data show that 26% of the affected population have the aa genotype as compared to 15% of the control population (Table II). Only 22% of the affected population had the A allele as compared to 34% of the control population. When the genotypes at the *Apa*I site of breast cancer individuals were compared with control individuals, again a significant difference was observed between the frequencies of each allele combination ($\chi^2 = 6.03$, df = 2, $p = 0.049$; not shown). The data support an additive effect of genotype (Fig. 1). These data indicate that the a allele may be associated with an increased risk of breast cancer, with those individuals of the aa genotype being at the greater risk. Conversely decreased risk may be associated with the AA genotype.

Allele frequencies of the *Taq*I polymorphism were compared between populations and a difference was observed ($\chi^2 = 3.76$, df = 1, $p = 0.053$). These results are indicative of a trend of increasing risk for breast cancer. A genotype comparison of the *Taq*I site between affected and control individuals, however, did not demonstrate a difference between frequencies of each allele combination ($\chi^2 = 4.33$, df = 2, $p = 0.115$). *Apa*I and *Taq*I genotypes are correlated (Morrison *et al.*, 1994) and combining *Apa*I and *Taq*I resulted in 5 of 9 possible combinations. Plotting the proportion of cancer cases in each *ApaI*/*Taq*I genotype (ignoring low abundance genotypes) was suggestive of a linear effect of genotype on cancer risk. Haplotypes were defined according to abundance: aT (haplotype 1), At (haplotype 2), at (haplotype 3) and a single genotype was observed compatible with haplotype AT (haplotype 4). Genotype aaTT was most strongly associated with cancer. There was a trend for increased risk of cancer across genotypes in the following order: AAtt (haplotypes 2,2), AATt (haplotypes 2,3), AaTt (haplotypes 1,2), aaTt (haplotypes 1,3) and aaTT (haplotypes 1,1). The data are consistent with haplotype 1 alone being associated with cancer. When haplotype 1 was tested using χ^2 against all other haplotypes (2, 3 and 4), a significant increase in haplotype 1 was observed in the cancer cases (Table III, $p = 0.029$). In particular, the genotype aaTT, representing homozygous haplotype aT, showed higher prevalence in cancer cases compared with controls. On a genotype basis, aaTT genotype had a 2.5-fold relative risk (95% CI: 1.02–6.5) of breast cancer when compared to Aatt.

TABLE II – COMPARISON OF ALLELE FREQUENCIES AMONG BREAST CANCER AFFECTED AND CONTROL POPULATIONS

Genotype	Breast cancer (n = 135)		Controls (n = 110)		χ^2 probabilities OR (95% CI)
	Count (%)	Frequency	Count (%)	Frequency	
*Apa*I					
AA	30 (22)	A 0.48	37 (34)	a 0.41	$p = 0.016$
Aa	70 (52)	a 0.52	56 (51)	A 0.59	1.56 (1.09–2.24)
aa	35 (26)		17 (15)		
*Taq*I					
TT	53 (39)	T 0.64	30 (27)	T 0.55	$p = 0.053$
Tt	67 (50)	t 0.36	62 (56)	t 0.45	1.45 (1.0–2.0)
tt	15 (11)		18 (17)		
*Fok*I					
FF	40 (30)	F 0.57	32 (31)	F 0.57	$p = 0.97$
Ff	74 (55)	f 0.43	55 (53)	f 0.43	0.99 (0.69–1.43)
ff	21 (15)		17 (16)		

Using a standard *t*-test, the average ages of the 2 populations were found to be significantly different ($p = 0.00$). In light of this, a simple factorial ANOVA was performed on the data. These results indicated that differences in age had no effect on the *Apa*I genotype in either population ($p = 0.44$). A logistic regression was also applied to the data using age as a covariate. *Apa*I genotypes were coded (as 1, 2, 3) and logistic regression of probability of cancer against genotype and age variables used to test if age could potentially confound the observed genotype associations. *Apa*I genotype association using this logistic regression indicated that the association was independent of the age of the subjects and still showed a significant association ($p = 0.04$). These data suggest that age differences in the test populations are not responsible for the observed *Apa*I genotype association.

Genotype frequencies suggested an increasing relative risk (RR) of cancer as the number of *Apa*I a alleles increased. When risk of the AA genotype was taken as 1.0, we observed that the OR of the Aa genotype was 1.54 (95% CI = 0.85–2.8) and that of the aa genotype was 2.54 (95% CI = 1.19–5.4). This is suggestive of increasing risk across genotypes and is consistent with an additive model of gene effect. In contrast, when allele frequencies of the *Fok*I polymorphism were compared between populations, no significant differences were observed. These results show that in this study population, the *VDR* initiation codon polymorphism is not associated with breast cancer whereas specific alleles in the 3′ region of the *VDR* gene identify an increased risk for breast cancer that is associated with the haplotype aT.

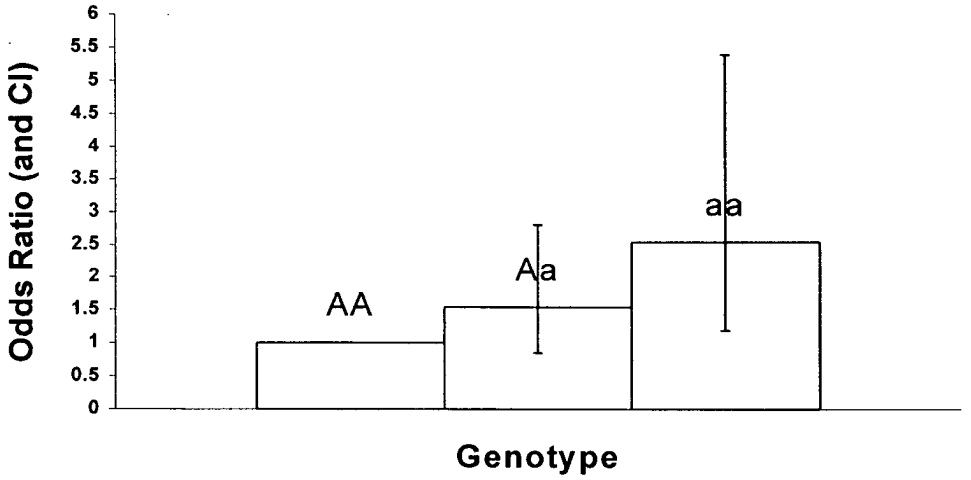

FIGURE 1 – Calculated relative risk associated with each genotype of the *ApaI VDR* polymorphism as compared with the AA genotype. OR results are presented as an average with 95% CI represented by bars for each genotype.

TABLE III – HAPLOTYPE COUNTS IN CANCER CASES AND CONTROLS

Haplotypes	n	*ApaI* and *TaqI*			
		1 (aT) (%)	2 (At) (%)	3 (at) (%)	4 (AT) (%)
Controls	108	89 (41)	97 (45)	30 (14)	0 (0)
Cancer	133	136 (51)	96 (36)	33 (12.5)	1 (0.5)

DISCUSSION

The hormonal form of vitamin D [1,25(OH)$_2$D3] functions to inhibit the proliferation and promote the differentiation of transformed cells in breast tissue and is mediated by the VDR, a transcription regulator (Colston, 1997). VDRs are present in normal human breast and other epithelial tissues as well as expressed in a proportion of breast tumor biopsies (Eisman *et al.*, 1979; Colston, 1997). Furthermore, low levels of 1,25(OH)$_2$D3 correlate with breast disease progression (Mawer *et al.*, 1997). Patients with VDR$^-$ tumors relapsed significantly earlier than those with VDR$^+$ tumors (Colston, 1997). Hence, the level of receptor expression in VDR$^+$ tumors and the circulating levels of the hormone could influence breast cancer cell growth. These in turn may be influenced by polymorphism in the *VDR* gene.

Our results suggest that alleles in the 3′ region of the *VDR* gene are associated with breast cancer risk. In a population of 135 female individuals affected by breast cancer and compared to a control population of 110 female individuals with no cancer history, the presence of the *ApaI* a allele or aa genotype was significantly associated with an increased risk for breast cancer development. In addition, the frequency of the T allele among the populations demonstrated a trend towards an increase in breast cancer risk. *ApaI* and *TaqI* genotypes are highly correlated in this and other populations (Morrison *et al.*, 1994; Carling *et al.*., 1997). Haplotype analysis suggested that a single haplotype, defined by *ApaI* and *TaqI* genotypes as aT, was associated with increased risk of breast cancer. In the present study, no association was seen between breast cancer and allele or genotype frequencies of the *FokI* polymorphism.

Previous studies have found polymorphisms of the *VDR* gene associated with several different cancer types. Ingles *et al.* (1997*a*,*b*) demonstrated that a poly-A sequence microsatellite in the 3′UTR region of *VDR* conferred a 4–5-fold increase in prostate cancer risk. Taylor *et al.*. (1996) found a similar risk of prostate cancer associated with the *TaqI* allele used in the present study. The TT genotype was associated with increased prostate cancer risk. Taylor *et al.* (1996) did not genotype for *ApaI* markers, however, the high co-association seen between *TaqI* and *ApaI* alleles suggests that the disease conferring haplotype in prostate cancer is the same that we have observed in breast cancer. Ruggiero *et al.* (1998) have reported an association of the *BsmI* bb genotype with increased risk of metastatic breast cancer. The *BsmI* marker is close by the *ApaI* marker and the bb genotype has high co-association with the aa genotype in Caucasian populations (Morrison *et al.*, 1994). These data reinforce the hypothesis that these markers are detecting the same risk causing allele on haplotype 1 (aT). Carling *et al.* (1997) reported an association of the T, b and a alleles of the *TaqI*, *BsmI* and *ApaI* polymorphisms with an increased risk for hyperparathyroidism and parathyroid adenoma (Carling *et al.*, 1995, 1997). These data could be generated by a genetic effect within the *VDR* gene itself or an unknown gene in sufficiently strong linkage disequilibrium with the *VDR* gene markers. Given that vitamin D analogues can limit cancer cell proliferation, it seems reasonable to speculate that the genetic association is generated within the *VDR* gene itself.

1,25(OH)$_2$D3 and analogues block the proliferation of breast cancer cells at G_1 in the cell cycle. VDR directly regulates p53 and p21^{waf}, suggesting a plausible mechanism whereby VDR protein levels may impact on cancer cell cycle progression (James *et al.*, 1996). Our present results suggest that studies involving VDR expression and binding in breast cancer patients may be an important future endeavor. Alternative exons in the 5′ region code for newly described protein variants that have a tissue-specific distribution (Crofts *et al.*, 1998). The initiation codon polymorphism is currently the only VDR variant that results in an alteration in the amino acid sequence and is intuitively attractive as a candidate for functional alteration. However, we found no relationship between the initiation codon polymorphism and breast cancer. Why would polymorphisms in the 3′ region be associated with several different types of cancer? At the 3′ end of *VDR* is a large UTR consisting of approximately 3,200 non-coding nucleotides that harbors several known sequence polymorphisms (Pike, 1997). The 3′UTR may control mRNA stability and differences in the total amount of VDR mRNA have been observed in some experiments (Crofts *et al.*, 1998). Another possibility is that the *VDR* gene markers are in disequilibrium with another gene that influences breast cancer.

Alleles of both VDR, *ApaI* and *TaqI*, polymorphisms appear to confer an increasing risk for breast cancer development, however, larger studies are warranted to confirm these results. Additional studies in varied breast cancer populations are also necessary to determine the true involvement of the VDR polymorphisms in the complex etiology of breast cancer.

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1. Breast Cancer Research and Treatment (2002), 74(1), 1-7
CODEN: BCTRD6; ISSN: 0167-6806

2. Proceedings of the American Association for Cancer Research Annual Meeting, (March, 2000) No. 41, pp. 129.
Meeting Info.: 91st Annual Meeting of the American Association for Cancer Research. San Francisco, California, USA April 01-05, 2000
ISSN: 0197-016X.

3. ONCOLOGY RESEARCH, (1998) 10 (1) 43-6.
Journal code: 9208097. ISSN: 0965-0407.

4.INTERNATIONAL JOURNAL OF CANCER, (1999 Dec 10) 83 (6) 723-6.
Journal code: 0042124. ISSN: 0020-7136.

5. CANCER CAUSES AND CONTROL, (2000 Jan) 11 (1) 25-30.
Journal code: 9100846. ISSN: 0957-5243.

6. BRITISH JOURNAL OF CANCER, (2001 Jul 20) 85 (2) 171-5.
Journal code: 0370635. ISSN: 0007-0920.

7.CARCINOGENESIS, (1999 Nov) 20 (11) 2131-5.
Journal code: 8008055. ISSN: 0143-3334.

THANK YOU

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Article title	Vitamin D receptor genotype and breast cancer in Latinas (United States)
Article identifier	0957524300105277
Authors	Ingles_S_A Garcia_D_G Wang_W Nieters_A Henderson_B_E Kolonel_L_N Haile_R_W Coetzee_G_A
Journal title	Cancer Causes and Control
ISSN	0957-5243
Publisher	Kluwer
Year of publication	2000
Volume	11
Issue	1
Supplement	0
Page range	25-30
Number of pages	6
User name	Adonis
Cost centre	
PCC	$21.50
Date and time	Saturday, July 27, 2002 2:30:07 AM

Cancer Causes and Control 11: 25–30, 2000.

Vitamin D receptor genotype and breast cancer in Latinas (United States)

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Received 5 November 1998; accepted in revised form 6 August 1999

Key words: breast neoplasms calcitriol, cohort studies, polymorphism (genetics), receptors.

Abstract

Objective: Polymorphism in the vitamin D receptor (VDR) gene has been associated with variation in bone mineral density and with prostate cancer risk. The purpose of this study was to determine whether polymorphism in the VDR gene may also influence breast cancer risk.

Methods: Polymorphisms in the 5′ and 3′ ends of the VDR gene were genotyped for 143 Latina women with breast cancer and 300 cohort controls.

Results: Both the BsmI and poly-A polymorphisms in the 3′ end of the VDR gene were associated with breast cancer risk, with a trend for increasing risk with increasing number of BsmI B alleles or short (S) poly-A alleles. Compared to subjects having two long poly-A alleles (genotype LL), odds ratios (and 95% confidence intervals) were 1.5 (1.0–2.3) and 3.2 (1.5–6.9) for subjects having genotypes SL and SS, respectively. Compared to BsmI genotype bb, odds ratios (and 95% confidence intervals) were 1.6 (1.1–2.5) and 2.2 (1.0–4.7) for genotypes Bb and BB respectively. The start codon polymorphism, FokI, was not associated with breast cancer risk.

Conclusion: These results suggest that polymorphic variation in or near the 3′ end of the VDR gene influences breast cancer risk in Latina women.

Introduction

Vitamin D plays an important role in modulating transcription of genes involved both in calcium and phosphorus homeostasis and in cellular differentiation and proliferation (reviewed in refs. 1–3). An obligatory mediator of these effects is the vitamin D receptor (VDR). Both the 5′ and 3′ ends of the VDR gene are polymorphic. Polymorphism in the first of two possible translation start codons [4] produces receptor variants differing in size and activity [5]. Allelic variation in the 3′ end of the VDR gene, although less clearly related to function, appears to have phenotypic consequences for calcium metabolism [6, 7], vitamin D metabolism [8, 9], bone density (reviewed in ref. 10) and prostate cancer risk [9, 11–13]. The observation that normal human breast epithelial cells [14] and most breast cancers [15, 16] express VDR raise the possibility that polymorphism in the VDR gene may also influence breast cancer risk. To test this hypothesis we genotyped polymorphisms in the 5′ and 3′ ends of the VDR gene for 143 Latina women with breast cancer and 300 cohort controls.

Materials and methods

Subjects

The Hawaii–Los Angeles Multiethnic Cohort Study is an ongoing epidemiological study of Japanese-Americans and whites residing in Hawaii, and African-Americans and Latinos residing in California. For a study of vitamin D receptor genotypes and breast cancer we obtained DNA samples on female subjects from the

two Los Angeles-based (*i.e.*, African-American and Latino) sub-cohorts. In this first report we focus on Latinas, a population with strong linkage disequilibrium in the genomic region of interest, which allows BsmI and poly-A genotypes to be interpreted as markers of the VDR 3'UTR allelotypes [17]. African-Americans have not been included in this report because characterization of allelotypes and validation of markers in the African-American population is still in progress.

The Latina sub-cohort includes more than 12,000 Latina women, age 45–75 at recruitment, residing in Los Angeles County, who were recruited by sampling Los Angeles County residents from drivers' license files as previously described [18, 19]. Newly diagnosed cases of breast cancer among Latinas were ascertained through linkage of the cohort to the Los Angeles County Surveillance, Epidemiology, and End Results (SEER) cancer registry, which is estimated to be 99% complete. Blood samples were obtained from incident breast cancer cases and from approximately a 1% random sample of the cohort members to serve as a control group. The participation rate for sample collection was in excess of 70% among both cases and controls. All subjects signed informed consents and the study was approved by the University of Southern California Institutional Review Board, which oversees studies involving human subjects.

Genotyping

An 825 bp region of genomic DNA containing the BsmI polymorphic site in intron 8 was amplified and analyzed as previously described [12]. The existence of the cut allele, b, is indicated by the formation of a 625 base-pair product.

A region surrounding the poly-A polymorphism was amplified as previously described [13]. Products were separated on polyacrylamide sequencing gels and autoradiographed. Alleles were sized and categorized as short (S) or long (L) as previously described [12].

A region of approximately 280 bp of genomic DNA containing the FokI polymorphic site was amplified and analyzed as previously described [20]. The presence of the cut allele, f, is indicated by a band at approximately 200 bp.

Statistical methods

FokI, BsmI and poly-A odds ratios were estimated by fitting standard unconditional logistic regression models [21], using two indicator variables to encode the three FokI, BsmI or poly-A genotypes, and two indicator variables to adjust for tertiles of age. Tests for trend were performed using a likelihood ratio test for significance of a genotype variable coded as 0, 1, or 2.

Expected genotype frequencies under Hardy–Weinberg equilibrium and under the hypothesis of no linkage disequilibrium were calculated from observed genotype frequencies among controls, using standard methods [22]. Testing for departure from the hypothesis of no linkage disequilibrium was performed by comparing observed and expected joint genotypic distributions using a chi-square test.

Results

Of the 300 control women, 55 were included in our previous report describing linkage disequilibrium in the 3'UTR of the VDR gene [17]. Among 55 female and 43 male Latino cohort members, we had observed 81% agreement between BsmI and poly-A genotypes. With 300 control women now available from the same cohort, agreement between the BsmI and poly-A genotypes is now estimated to be 88% (Table 1).

Table 1. Joint distribution of 3'VDR marker genotypes (BsmI and Poly-A) among Latina controls

	Poly-A			
	LL	SL	SS	Total
BsmI				
bb	12	7	0	19
Bb	4	94	14	112
BB	0	12	157	169
Total	16	113	171	300

Agreement = (12 + 94 + 157)/300 = 88%.

Table 2. Age-adjusted odds ratios and 95% confidence intervals for breast cancer by 3'VDR genotype in Latinas

	Controls *n* (%)	Cases *n* (%)	OR (95% confidence interval)
BsmI			
bb	169 (56.4)	61 (42.7)	1.0
Bb	112 (37.3)	68 (47.5)	1.6 (1.1–2.5)
BB	19 (6.3)	14 (9.8)	2.2 (1.0–4.7)
			p-trend = 0.01
Poly-A			
LL	171 (57.0)	62 (43.4)	1.0
SL	113 (37.7)	65 (45.4)	1.5 (1.0–2.3)
SS	16 (5.3)	16 (11.2)	3.2 (1.5–6.9)
			p-trend < 0.01
Total	300 (100)	143 (100)	

Both BsmI and poly-A genotype frequencies were in Hardy–Weinberg equilibrium among the cohort controls, with observed genotype frequencies (Table 2) being nearly identical to the expected Hardy–Weinberg frequencies of 57%, 37%, and 6% for genotypes bb or LL, Bb or SL, and BB or SS, respectively. Cases, on the other hand, had a lower than expected frequency of bb or LL and higher than expected frequencies of Bb or SL and BB or SS genotypes. Thus 3'VDR genotype, as measured by either the BsmI or the poly-A marker, was associated with breast cancer risk (Table 2). Compared to subjects having the BsmI bb genotype, risk was increased by approximately 60% in heterozygotes (Bb) and more than two-fold in BB homozygotes. Similar results were obtained using poly-A as a marker of 3'VDR genotype.

The start codon polymorphism, FokI, was not in linkage disequilibrium with the BsmI and poly-A polymorphisms in the 3' end of the gene. The observed joint distribution of FokI and BsmI genotypes did not differ significantly from the expected distribution under the hypothesis of no linkage disequilibrium (Table 3), indicating that the two ends of the VDR gene segregate independently, or nearly so in this population. Moreover, the FokI polymorphism was not associated with breast cancer risk (Table 4). Genotype frequencies were similar for cases and controls, and odds ratios comparing genotypes Ff and ff to FF were not significantly different from the null hypothesis value of 1.

Table 3. Joint distribution of 5'VDR (FokI) and 3'VDR (BsmI) genotypes among Latina controls: observed frequencies (and expected frequencies under the assumption of no linkage disequilibrium)

	BsmI		
	bb	Bb	BB
FokI			
FF	51 (61)	45 (40)	11 (7)
Ff	89 (81)	50 (54)	8 (9)
ff	29 (27)	17 (18)	0 (3)

Test for departure from hypothesis of no linkage disequilibrium: $p = 0.21$.

Table 4. Age-adjusted odds ratios and 95% confidence intervals for breast cancer by 5'VDR genotype in Latinas

	Controls *n* (%)	Cases *n* (%)	OR (95% confidence interval)
FokI			
FF	107 (35.7)	53 (37.1)	1.0
Ff	147 (49.0)	65 (45.5)	0.9 (0.6–1.4)
ff	46 (15.3)	25 (17.5)	1.1 (0.6–2.9)
Total	300 (100)	143 (100)	

Of the 143 cases, 22 (15%) were diagnosed with *in-situ* disease, 86 (60%) with stage 1 disease, and 35 (25%) with higher-stage disease. Results did not appear to differ by stage; however, power to detect heterogeneity was low. The average age was 65.5 (s.d. = 8.3) years for cases and 62.7 (s.d. = 7.9) years for controls.

Discussion

In this cohort of Latina women, the frequency of the BsmI b allele, at 75%, is higher than frequencies typically reported for non-Latino white populations (*e.g.*, 59% among 591 control subjects in the Physician's Health Study [9]; 57% among 169 non-Latino white control subjects residing in Los Angeles County [17]). However, the BsmI b allele frequency among our Latina control subjects is similar to the frequency of 71% observed among 103 Mexican-American women in an observational study of fracture risk in Northern California [23]. Although the Northern California study was not population-based, any bias due to selection of women at increased fracture risk would be expected to produce downward bias in the frequency of the BsmI b allele, which is associated with lower fracture risk. The BsmI b allele frequency among Latinos, at 71–75%, is lower than frequencies typically reported in Asian populations (*e.g.*, 88% among 488 healthy premenopausal Japanese women [24]; 95% among 96 members of a Singapore cohort [17]). The observation of BsmI allele frequencies in Latinos that are intermediate between non-Latino whites and Asians is consistent with the known ethnic make-up (part European, part native-American) of this population.

In this cohort of Latina women, breast cancer risk was associated with polymorphic variation in the 3' end of the VDR gene. The VDR, a transcription factor which regulates a number of genes involved in cell proliferation and differentiation (reviewed in refs 1–3), was first observed in human breast cancer cells nearly 20 years ago [25]. Since that time, numerous studies have demonstrated that vitamin D and deltanoids (vitamin D analogues) inhibit proliferation of breast cancer cells both *in vitro* [25–33] and *in vivo* [15, 30–32, 34–36]. The anti-proliferative effect is confined to those cells possessing VDRs [28, 37], and is roughly proportional to VDR number [37].

The simplest hypothesis that might explain an association between breast cancer risk and 3'VDR genotype is that two allelic variants encode receptors differing in steady-state expression or functional activity. Tests of this hypothesis, however, have yielded conflicting results, with VDR gene expression having been reported

as: higher for BsmI B compared to b alleles [8, 38]; higher for b compared to B alleles [39]; and indistinguishable for B and b alleles [40, 41]. Contributing to these contradictory results may be the use of markers, such as BsmI, which do not lie in the 3'UTR itself, to classify a relatively small number of cell lines in all of these studies. Although BsmI can be used as a marker of 3'UTR polymorphisms in most populations [17], it cannot be presumed that the B allele, for example, is in *cis* with the functional allele of interest in any single cell line. Potentially functional polymorphisms in the 3'UTR itself have not yet been tested in *in vitro* systems. Based on *in vitro* studies reported to date, the VDR 3'UTR cannot be ruled out as a functional locus contributing to breast cancer risk.

At the 5' end of the VDR gene, the start codon polymorphism, FokI, was not associated with breast cancer risk. Thus, the association between breast cancer and the 3'VDR genotype is not due to linkage disequilibrium between the 3' end of the gene and the start codon polymorphism. Sequences upstream of the start codon are also unlikely candidates for the functional locus, since the start codon polymorphism lies outside the region of tight linkage disequilibrium surrounding the BsmI polymorphism. The region of disequilibrium extends at least 3 kb downstream from BsmI to the poly-A microsatellite approximately 1 kb from the end of the VDR 3'UTR [17], and may extend further downstream to include other genes. Thus, we cannot rule out the possibility that BsmI and poly-A are markers for a nearby downstream gene.

The association of 3'VDR genotype with variation in traits that are clearly dependent on vitamin D status, such as calcium metabolism and bone mineral density, lends support to the hypothesis that polymorphism within the VDR gene itself is functionally significant. The BsmI BB genotype, which we found to be associated with increased risk of breast cancer, has been associated with decreased bone density in most studies [42]. It can be hypothesized that variation in the VDR gene marked by the BsmI B allele, either by affecting VDR activity or VDR number, leads to decreased *trans*-activation of VDR target genes that influence calcium metabolism or cellular growth or proliferation. Likely target genes include genes for insulin-like growth factor (IGF) binding proteins, which in addition to regulating bone formation, may mediate growth inhibitory effects of vitamin D in breast cancer cell lines [43, 44]. Although breast cancer and osteoporosis have been found to be inversely correlated at the population level [45, 46], this may be explained by the role of estrogen both in maintaining bone mineral density and in driving cellular proliferation in the breast. The relative contributions of estrogen and vitamin D status to risk of breast cancer and osteoporosis, as well as possible interactions among estrogen, vitamin D, and the IGF system, need to be further studied.

Finally, we note that the BsmI b and poly-A L alleles that were associated with protection against breast cancer were previously found to be associated with increased risk of prostate cancer [9, 12]. While the reason for this finding is not yet clear, it is not surprising that a steroid hormone such as $1,25(OH)_2D_3$ may have different effects in different tissues. The VDR can act both as an activator and as a repressor of transcription, depending on the nature of the target gene promoter and on tissue-specific VDR interacting proteins [47]. The specific target genes and regulatory factors involved in breast- and prostate-specific VDR responses have not been identified; nevertheless, even in the absence of mechanistic explanations, the finding of an association between VDR genotype and breast cancer risk supports the hypothesis that vitamin D may influence breast cancer etiology. However, because the sequence variants defining the functional 3'VDR genotype have not yet been identified, it is especially important that this epidemiologic finding is replicated in other populations and in other ethnic groups. If confirmed, these findings suggest that vitamin D and/or vitamin D analogues may be useful for breast cancer prevention and/or treatment, and that assessment of VDR polymorphisms might someday be useful to identify individuals most at risk and/or most responsive to intervention.

Acknowledgements

We thank Wu Zhang for laboratory assistance and Hank Hwang for programming assistance. This study was supported by funds from the California Breast Cancer Research Program of the University of California, Grants number 1IB-0353 and 3IB-0089 and by NIH/NCI grant R01 CA54281. Dr Ingles was supported by the STOP Cancer Foundation.

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2. Proceedings of the American Association for Cancer Research Annual Meeting, (March, 2000) No. 41, pp. 129.
Meeting Info.: 91st Annual Meeting of the American Association for Cancer Research. San Francisco, California, USA April 01-05, 2000
ISSN: 0197-016X.

3. ONCOLOGY RESEARCH, (1998) 10 (1) 43-6.
Journal code: 9208097. ISSN: 0965-0407.

4.INTERNATIONAL JOURNAL OF CANCER, (1999 Dec 10) 83 (6) 723-6.
Journal code: 0042124. ISSN: 0020-7136.

5. CANCER CAUSES AND CONTROL, (2000 Jan) 11 (1) 25-30.
Journal code: 9100846. ISSN: 0957-5243.

6. BRITISH JOURNAL OF CANCER, (2001 Jul 20) 85 (2) 171-5.
Journal code: 0370635. ISSN: 0007-0920.

7.CARCINOGENESIS, (1999 Nov) 20 (11) 2131-5.
Journal code: 8008055. ISSN: 0143-3334.

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British Journal of Cancer (2001) 85(2), 171–175
© 2001 Cancer Research Campaign
doi: 10.1054/ bjoc.2001.1864, available online at http://www.idealibrary.com on IDEAL®

Vitamin D receptor gene polymorphisms are associated with breast cancer risk in a UK Caucasian population

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Summary There is increasing evidence that vitamin D can protect against breast cancer. The actions of vitamin D are mediated via the vitamin D receptor (VDR). We have investigated whether polymorphisms in the VDR gene are associated with altered breast cancer risk in a UK Caucasian population. We recruited 241 women following a negative screening mammogram and 181 women with known breast cancer. The VDR polymorphism *Bsm*I, an intronic 3′ gene variant, was significantly associated with increased breast cancer risk: odds ratio *bb* vs *BB* genotype = 2.32 (95% CI, 1.23–4.39). The *Bsm*I polymorphism was in linkage disequilibrium with a candidate translational control site, the variable length poly (A) sequence in the 3′ untranslated region. Thus, the 'L' poly (A) variant was also associated with a similar breast cancer risk. A 5′ VDR gene variant, *Fok*I, was not associated with breast cancer risk. Further investigations into the mechanisms of interactions of the VDR with other environmental and/or genetic influences to alter breast cancer risk may lead to a new understanding of the role of vitamin D in the control of cellular and developmental pathways. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: vitamin D; receptor; human; polymorphism; breast cancer; caucasian

Breast cancer is the commonest cancer among women in the UK, with a lifetime risk of almost 1 in 10 (Anderson, 1992). While familial cancers account for around 5% of all breast cancers, the remainder appear to be the result of a multifactorial aetiology that includes a genetic component. Breast cancer is known to be strongly influenced by the hormonal milieu, and variation in genes that are responsive to such hormones are therefore possible candidates for increased risk. One potential target is the vitamin D receptor (VDR), a member of the steroid-hormone family of nuclear receptors, which are responsible for the transcriptional regulation of a number of hormone-responsive genes. The vitamin D receptor (VDR) is expressed in breast tissue, and patients with VDR-positive breast tumours have longer disease-free survival compared to those with receptor-negative tumours (Colston et al, 1989). The VDR ligand is the vitamin D metabolite, 1,25 dihydroxyvitamin D_3 (1,25-D), which has potent effects on cell growth and differentiation. Laboratory studies have demonstrated that 1,25-D and its analogues inhibit cell proliferation and promote apoptosis in breast cancer cells in culture (Chouvet et al, 1986; Eisman et al, 1989; James et al, 1995; Welsh, 1995). In animal models of breast cancer, vitamin D analogues delay tumour development and cause regression of established mammary tumours (Abe et al, 1991; James et al, 1998). Such evidence has led to the development of vitamin D analogues as potential new therapeutic agents (Bower et al, 1991; Gulliford et al, 1998) in breast cancer.

The gene encoding the VDR is known to contain a number of polymorphisms. A polymorphic start codon in the 5′ end of the gene (identified by the restriction enzyme *Fok*I) results in VDR proteins that differ in length by 3 amino acids. This polymorphism has been associated with increased breast cancer risk in African-American women (Ingles et al, 1997a). 3 sequences in the 3′ end of the gene (generating *Bsm*I, *Apa*I and *Taq*I restriction sites) are thought to be linked to a further polymorphism, the variable length poly (A) sequence in the 3′ untranslated region (3′ UTR). Such 3′UTR elements are candidate translational control sites, important in the post-transcriptional control of gene expression (Day and Tuite, 1998). An association between these 3′ polymorphisms and bone mineral density has been widely reported (Morrison et al, 1994; Cooper and Umbach, 1996). They have also been related to a number of other diseases including prostatic and breast carcinoma: in separate US studies, increased risk of prostate cancer has been associated with a long poly (A) allele (Ingles et al, 1997b), absence of *Taq*I (Taylor et al, 1996) and presence of *Bsm*I (Ma et al, 1998) restriction sites. An association has also been reported between breast cancer risk and the *Apa*I polymorphism (Curran et al, 1999), breast cancer progression and absence of the *Taq*I polymorphism (Lundin et al, 1999), and *Bsm*I genotype and increased risk of breast metastases (Ruggiero et al, 1998). These polymorphisms are thought to be in linkage disequilibrium in Caucasian populations, suggesting they are essentially looking at the same genotype (Ingles et al, 1997c).

This study was undertaken to assess whether VDR polymorphisms in both the 3′ and 5′ end of the gene are associated with breast cancer risk in a UK Caucasian population. In order to be as certain as possible that our control group is free of any breast cancer or precancerous changes that may go undetected in the general population, women were only recruited following a negative screening mammogram.

Received 18 October 2000
Revised 26 March 2001
Accepted 27 March 2001

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METHODS

Subjects

For both sample groups, written informed consent was obtained at the time of interview and sampling. The study was approved by the

St George's Hospital Medical School Ethics Committee. Recruitment criteria indicated that volunteers (i) were Caucasian and (ii) had had a recent mammogram. Women with family history of breast cancer were not specifically excluded from the study: the reported incidence was not different between our control and case groups and showed no relationship with VDR genotype.

Control volunteers

Women (n = 241) were recruited through the UK National Breast Screening Programme for South-West London. This provides for routine mammography of all women between the ages of 50–65 years at 3 yearly intervals, and 65+ years by self-referral. Currently, around 5% women screened are recalled to the Unit due to a technical problem with their mammogram, or for further investigation. Most of these women are subsequently found to be healthy and are discharged back into the Screening Programme. These women therefore have no detectable cancer at time of sampling, although 89 women had breast conditions not associated with breast cancer risk (26 had benign calcifications, 26 had fibrocystic disease and 37 had a fibroadenoma/other benign lump). The benign nature of these conditions was confirmed by cytology and/or radiological stability over time. Because of the nature of the Screening Programme, control women were in the age range 50–81 years, with a median age of 55.2 years, at the time of sampling. The age of the control group was therefore different from the case group. However, there were no age-related differences in VDR gene frequency between the oldest and youngest women. The majority of the control group (167 women, 69%) were postmenopausal and 126 women (52%) were current or past users of hormone replacement therapy (HRT).

Breast cancer volunteers

Women (n = 181) were recruited through the Combined Breast Clinic at St. George's Hospital. Women had a median age of 62.1 (range 29.0–91.0) years at the time of sampling. The median age at diagnosis was 57.2 (range 26.2–89.8) years, with a median time since diagnosis of 4.3 (range 0.4–27.5) years. Of this group, 149 women (82%) were postmenopausal, and 52 (29%) women were current/past users of HRT. All women had a surgical procedure (wide local excision or mastectomy) with or without post-operative radiotherapy.

The characteristics of the tumours were confirmed from histopathological records of core biopsy and/or resection specimens: 20 women had ductal carcinoma-in-situ (DCIS), 70 invasive ductal carcinoma (IDC), 77 both DCIS and IDC, and 14 invasive lobular carcinoma with or without lobular carcinoma in situ. Tumour grade data was available for 147 patients: 47 (32%) patients were classed as Bloom and Richardson Grade I, 58 (39%) patients as Grade II and 42 (29%) patients Grade III. Lymph nodes were taken from 140 patients. Of these, 108 (77%) had no lymph node involvement. Oestrogen receptor (ER) levels were determined for 167 patients, of whom 131 (78%) were ER positive. Adjuvant tamoxifen was given to 108 women, adjuvant chemotherapy to 20 women and a combination of both to 32 women. Since diagnosis, 21 women have had local recurrence, 3 women have had new primary breast tumours and 4 have developed metastatic disease.

Analysis of VDR polymorphisms

A 10 ml blood sample was collected into a lithium heparin tube and used for extraction of DNA (QIAamp blood kit, Quiagen UK Ltd, W Sussex, England). Genomic DNA was amplified by PCR using specific primers as previously described (Morrison et al, 1994; Ingles et al, 1997b; Gross et al, 1998). For *Bsm*I and *Fok*I genotyping, PCR product was digested with the appropriate restriction endonuclease (New England Biolabs UK Ltd, Hertfordshire, England), separated by agarose gel electrophoresis and visualized by ethidium bromide staining. For both *Bsm*I and *Fok*I, genotypes were defined by capital letters in the absence of the restriction site (*B, F* respectively) and small letters where the restriction site was present (*b, f*). For the poly (A) analysis, a 425 base pair PCR product was separated on a 6% PAGE-urea gel and visualized by silver staining (Silver Sequence^R, Promega UK, Southampton, England). Under these conditions, the poly (A) region resolves into 2 distinct populations, long (L, A18–A24) and short (S, A13–A17).

Statistical analysis

The χ^2 test was used to assess any association between VDR polymorphisms and breast cancer risk. Odds ratios and 95% confidence intervals were calculated to determine the risk of breast cancer associated with a given VDR genotype using the Clinistat Programme, devised by Professor Martin Bland, Dept. Public Health, St George's Hospital Medical School, London, UK. Allele frequencies were assessed for deviation from expected Hardy-Weinberg Equilibrium using the χ^2 test.

RESULTS

The frequency of VDR polymorphisms in our sample populations are shown in Table 1. We found a highly significant difference in genotype frequencies between patients and controls, such that the *bb* genotype was significantly over-represented in the patient population (Table 1). The odds of breast cancer for a woman of genotype *bb* were twice (OR = 2.32, 95% CI 1.23–4.39, Table 1) those for a woman of genotype *BB*.

The allele frequencies were similar to those reported in other Caucasian populations (Morrison et al, 1994; Houston et al, 1996; Harris et al, 1997; Ingles et al, 1997b; Kiel et al, 1997; Vandevyer et al, 1997; Ferrari et al, 1998). There was slight deviation from expected Hardy-Weinberg frequencies (P = 0.05) for the *Bsm*I and poly (A) genotypes, but not the *Fok*I, in the control population only. Review and checking of our genotyping, including sequencing 12 random control DNA samples around the *Bsm*I site (data not shown), confirmed our genotyping. As the *Fok*I polymorphism was in Hardy-Weinberg equilibrium, we suggest that this result is an anomaly of our sample group.

The *Bsm*I polymorphism was found to be in strong linkage disequilibrium (410/419 samples genotyped, 98% agreement) with the variable length poly (A) sequence, such that *bb* genotype co-segregated with LL long poly (A). Independent analysis of breast cancer risk associated with the poly (A) genotype were similar to those associated with *Bsm*I (Table 1). No association was found between *Fok*I genotype and breast cancer risk (Table 1). The *Fok*I genotype was not in linkage disequilibrium with the poly (A) polymorphism (data not shown), suggesting that they segregate independently.

Table 1 VDR polymorphism frequencies

	Controls *n* (%)	Allele frequency	Cases *n* (%)	Allele frequency	Odds ratio (95% confidence interval)
BsmI					χ^2 test, $P = 0.0061$** (d.f.=2)
bb	69 (28.6)	*b* 0.56	78 (43.1)	*b* 0.66	2.32 (1.23–4.39)
Bb	133 (55.2)	*B* 0.44	84 (46.4)	*B* 0.34	1.30 (0.70–2.39)
BB	39 (16.2)		19 (10.5)		1.0
Poly A					χ^2 test, $P = 0.0068$** (d.f.=2)
LL	67 (28.2)	L 0.56	76 (42.0)	L 0.66	2.46 (1.29–4.70)
LS	132 (55.5)	S 0.44	87 (48.1)	S 0.34	1.43 (0.77–2.66)
SS	39 (16.4)		18 (9.9)		1.0
FokI					χ^2 test, $P = 0.68$ (d.f.=2)
FF	86 (35.7)	*F* 0.60	72 (39.8)	*F* 0.62	1.17 (0.65–2.08)
Ff	116 (48.1)	*f* 0.40	81 (44.8)	*f* 0.38	0.97 (0.55–1.71)
ff	39 (16.2)		28 (15.5)		1.0

** statistically significant. d.f. = degrees of freedom.

We investigated whether any of the VDR polymorphisms were associated with particular clinical/pathological characteristics of our breast cancer group (Table 2). Data is shown only for *Bsm*I polymorphism, although all 3 genotypes were tested. Due to the small numbers of *BB* genotype, for χ^2 test analysis data were pooled from *BB* and *Bb* genotypes. There was no association between any VDR genotype and either ER expression of the tumour or lymph node involvement. However, there was a significant association between the tumour grade and *Bsm*I genotype, with an excess of *bb* genotype in those tumours of grades II and III. Similar results were seen for poly (A) genotype (results not shown). This suggests that the *Bsm*I/poly (A) is associated with tumour progression in addition to disease risk.

DISCUSSION

We have found a highly significant association between the risk of breast cancer and the 3′ VDR gene polymorphisms, *Bsm*I and variable length poly (A) microsatellite, in a UK Caucasian population. This study has added to the increasing evidence for a role of VDR gene polymorphisms in the disease process: polymorphisms have been widely associated with disorders of bone (Morrison et al, 1994; Cooper and Umbach, 1996), and there is increasing evidence for an association with prostate and breast cancer (Taylor et al, 1996; Ingles et al, 1997b; Ma et al, 1998; Ruggiero et al, 1998; Curran et al, 1999; Lundin et al, 1999).

In Caucasian populations, there is thought to be strong linkage disequilibrium between the 3′ polymorphisms, *Bsm*I, *Apa*I, *Taq*I and the variable length poly (A), such that only 2 haplotypes are commonly observed: *baTL* and *BAtS* (Morrison et al, 1994; Ingles et al, 1997c). It is the *baTL* haplotype that appears to be associated with increased risk of breast and prostate cancer (Taylor et al, 1996; Ingles et al, 1997b; Ma et al, 1998; Ruggiero et al, 1998; Curran et al, 1999; Lundin et al, 1999), while the *BAtS* haplotype is associated with increased risk of osteoporosis (Morrison et al, 1994; Cooper and Umbach, 1996). Not all studies have shown such associations: increased breast cancer risk among Latina women in the United States was associated with SS/*BB* genotypes (Ingles et al, 2000), and no association was found between the VDR *Taq*I polymorphism and breast cancer risk in Caucasian women in the UK (Dunning et al, 1999).

There are difficulties, however, in equating different polymorphisms between studies. For example, the strength of linkage disequilibrium can vary significantly between populations (Ingles et al, 1997c), resulting in misclassification of the 'at-risk' locus. VDR allele frequencies may also vary within Caucasian populations: of

Table 2 Further analysis of cancer group in relation to *Bsm*I genotype

Risk factor				χ^2 test for *Bsm*I genotype
ER status of tumour	*ER+ve*	*ER–ve*		
bb	59	14		χ^2 test, $P = 0.51$ (d.f.=1)
Bb/BB	*72*	*22*		
total	131	36		
Lymph node involvement	*Node –ve*	*Node+ve*		
bb	45	15		χ^2 test, $P = 0.60$ (d.f.=1)
Bb/BB	*63*	*17*		
total	108	32		
Tumour Grade	*Grade I*	*Grade II*	*Grade III*	
bb	15	32	16	χ^2 test, $P = 0.043$* (d.f.=2)
Bb/BB	*32*	*26*	*26*	
total	47	58	42	

* statistically significant. d.f. = degrees of freedom.

10 polymorphic genes assessed in a Finnish study, the prevalence of the 3′ *Taq*I polymorphism was significantly different from other Caucasian populations (Woodson et al, 1999). That the Caucasian population is heterogeneous with respect to the VDR gene is one possible explanation for such discrepancies between population-based VDR association studies.

Another difficulty with VDR polymorphisms studies is that, to date, no clearly defined functional effect of the different genotypes has been demonstrated. The *BB* genotype has been associated with elevated serum calcitriol levels (Morrison et al, 1994; Ma et al, 1998), and the *BAt* haplotype shown to have increased activity in a reporter gene assay (Morrison et al, 1994). There has been no conclusive demonstration of any genotype association with VDR mRNA levels: no effect (Mocharla et al, 1997; Gross et al, 1998), decreased (Carling et al, 1998) and increased (Verbeek et al, 1997) expression of mRNA have all been reported.

One problem is the nature of the polymorphisms: the *Bsm*I and *Apa*I polymorphisms are intronic, while *Taq*I leads to a silent codon change. As such, they do not apparently lead to any change in either the transcribed mRNA or translated protein. It has been suggested that they are markers for the poly (A) sequence: such elements in the 3′ UTR of genes are thought to be important in post-transcriptional control of gene expression (Day and Tuite, 1998), either by altering mRNA stability or the interaction of the mRNA with the translational apparatus. There has been no evidence to date, however, that the poly (A) element has such a role – attempts to demonstrate an effect on mRNA stability were negative (Durrin et al, 1999). Further work is clearly necessary to try and identify functional mechanisms for the observed effects of the VDR polymorphisms.

Finally, while VDR polymorphisms may influence disease risk, such genetic factors cannot be dissociated from environmental influences. Breast cancer is strongly influenced by hormonal factors, in particular oestrogen. We found no association between VDR genotype and ER status in our group of largely post-menopausal women. In contrast, Lundin et al (1999) found a trend towards increased survival in ER-positive, pre-menopausal women possessing the *tt* genotype. However, the relationship between VDR polymorphisms and breast cancer risk may be very different in pre- and post-menopausal women. Dietary influences may also be important in modulating risk: *bb* genotype has been associated with increased prostate cancer in elderly men who are also deficient in vitamin D (Ma et al, 1998). It would be interesting to determine the extent of vitamin D deficiency in conjunction with VDR polymorphisms and breast cancer risk.

In conclusion, this study has provided additional support for a significant association between VDR gene polymorphisms and the risk of breast cancer. The potent actions of vitamin D and its analogues as anti-proliferative and pro-differentiation agents in breast cancers (Chouvet et al, 1986; Eisman et al, 1989; Abe et al, 1991; James et al, 1995; Welsh, 1995; James et al, 1998) have led to the suggestion that endogenous levels of active vitamin D are a factor in the development and progression of breast cancers. However, the ability of 1,25-D to act at the cellular level will be influenced by levels and activity of the VDR. While vitamin D and its analogues are being developed as preventative and/or treatment agents in breast cancer, the assessment of VDR polymorphisms may be vital in the identification of at-risk groups and strategies for targeting and intervention.

ACKNOWLEDGEMENTS

This research was supported by the St George's Hospital Special Trustees Research Fund. We would like to acknowledge Professor Martin Bland, Dept Public Health, St George's Hospital Medical School, London and Dr EL Duncan, Wellcome Trust Centre for Human Genetics, Oxford for their statistical advice and discussions. We are grateful to Claire MacDonald for assisting with sample collection and Dr Petros Syrris for the DNA sequencing.

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From: Goldberg, Jeanine
Sent: Friday, July 26, 2002 12:09 PM
To: STIC-ILL
Subject: please pull vdr poly breast cancer

1. Breast Cancer Research and Treatment (2002), 74(1), 1-7
   CODEN: BCTRD6; ISSN: 0167-6806

2. Proceedings of the American Association for Cancer Research Annual Meeting, (March, 2000) No. 41, pp. 129.
   Meeting Info.: 91st Annual Meeting of the American Association for Cancer Research. San Francisco, California, USA April 01-05, 2000
   ISSN: 0197-016X.

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   Journal code: 8008055. ISSN: 0143-3334.

THANK YOU

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Carcinogenesis vol.20 no.11 pp.2131–2135, 1999

No association between androgen or vitamin D receptor gene polymorphisms and risk of breast cancer

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Endogenous hormone exposure is known to alter breast cancer susceptibility and genes responsive to such hormones are plausible candidates for predisposition genes. We have examined polymorphisms in genes for two members of the nuclear receptor superfamily which are expressed in breast tissue and known to moderate rates of cell proliferation in a case–control association study: the androgen receptor (AR) and the vitamin D receptor (VDR). We have used two series of Caucasian female breast cancer cases, one incident and one prevalent, and compared both with two sets of matched controls from the East Anglian region of Britain. Since the results are similar in the two series we have combined them. The AR poly[Gly]$_n$ and poly[Gln]$_n$ tracts were genotyped in a total of 508 female breast cancer cases and 426 controls. The VDR *Taq*I polymorphism was analysed in 951 cases and 627 controls drawn from the same population series. There were no significant differences between cases and controls for either the AR or VDR polymorphisms. Compared with individuals with two short alleles (<22 repeats) of the AR poly[Gln]$_n$ tract, the odds ratios and 95% confidence intervals (95% CI) for individuals with one or two long alleles were 0.82 (95% CI 0.62–1.09) and 1.31 (95% CI 0.87–1.97), respectively. Heterozygotes and homozygotes for the VDR *Taq*I cutting site had odds ratios of 1.01 (95% CI 0.81–1.27) and 0.97 (95% CI 0.71–1.32), respectively. None of the AR or VDR polymorphisms investigated has a major effect on risk of breast cancer in the British population.

Introduction

Breast cancer is a common disease with major public health implications. Rare highly penetrant mutations in genes such as BRCA1, BRCA2 and TP53 explain <5% of total incidence. It is likely that other susceptibility genes exist: the first degree relatives of breast cancer cases have a 2-fold increase over the general population risk and only ~15% of this excess can be explained by known genes (1). These presently unidentified genes are likely to include commoner low penetrance predisposition alleles that could in principle explain a much greater proportion of total disease incidence than rare mutations of higher penetrance. To date there is substantial evidence for only one such locus, the HRAS1 VNTR (variable number of tandem repeats). This locus has a number of rare alleles, with a combined population frequency of 6%, which are associated with a 2-fold increased relative risk of breast cancer (2). Despite this small increase in risk, these alleles are together calculated to account for ~9% of total breast cancer incidence.

Breast cancer risk is known to be strongly related to endogenous hormone exposure and genes responsive to such hormones are therefore plausible candidates for being susceptibility genes. The androgen receptor (AR) and vitamin D receptor (VDR) are both members of the nuclear receptor superfamily which regulate the action of hormone-responsive genes. They function by binding hormone, androgens or 1,25-dihydroxyvitamin D, respectively, and then transactivating their respective hormone-responsive genes via hormone response elements in the promotors of the downstream genes. Both receptors are expressed in breast tissue and are known to moderate rates of cell division.

Missense mutations in the AR have been shown to cause partial androgen insensitivity (leading to feminization, including gynaecomastia) together with familial male breast cancer in a few families (3,4). Two of the amino acid substitutions implicated, Arg607Gln and Arg608Lys in the DNA-binding domain, reduce the affinity of these mutant ARs for their response elements and thus their transactivation efficiencies (5). The AR is expressed in female breast epithelium and androgens binding to these receptors inhibit breast cell proliferation (6,7), although AR status in breast tumours is not so strongly correlated with prognosis as oestrogen receptor and progesterone receptor status (8).

Exon 1 of the AR encodes two expressed polymorphic repeats, a poly[Gly]$_n$ and a poly[Gln]$_n$ tract. Expansion of the poly[Gln]$_n$ tract beyond the normal range of 11–35 repeats causes X-linked spinal-bulbar muscular atrophy (Kennedy's disease) and frequently also mild androgen insensitivity (9). The length of the poly[Gln]$_n$ tract has been shown to be inversely related to transactivation efficiency and rate of sperm production (10,11). Recently, some studies have suggested an association of the poly[Gln]$_n$ tract with differences in prostate cancer risk: the shorter, more activating, repeat lengths are associated with a mildly increased (1.5- to 2.0-fold) risk of prostate cancer or an earlier age of onset and possibly also an increased risk of metastasis (12–16). The poly[Gly]$_n$ tract has also been investigated (17), but shows no significant differences between prostate cases and controls. Elhaji *et al.* (18) have also compared the poly[Gln]$_n$ tract in 80 breast tumour DNA samples with control germline DNA. They found longer repeat lengths in tumours than in the controls but their study design was unable to differentiate between somatic mutation in the tumour and the patient's germline genotype. Rebbeck *et al.* (19) have studied this polymorphism in carriers of BRCA1 mutations, who are thus at very high risk of developing breast

Abbreviations: AR, androgen receptor; l, long; PAR, population attributable risk; RFLP, restriction fragment length polymorphism; s, short; VDR, vitamin D receptor.

cancer, and they have reported an earlier age of onset of cancer in individuals with >28 Gln (less activating) repeat lengths. Finally, Wu *et al.* (20) have very recently reported that 81 male breast cancer cases have a mean of 28 ± 3 repeats compared with 22 ± 3 in 73 normal male controls.

The VDR is expressed in breast cell lines. The hormonal form of vitamin D (1,25-dihydroxyvitamin D) has antiproliferative effects on breast tumour cells (21). There are numerous reported polymorphisms in the VDR gene: three, detectable with the enzymes *Bsm*I, *Apa*I and *Taq*I, located in intron 8 and exon 9, and others in the 3′-UTR (22). Three large studies all show these to be in strong linkage disequilibrium in Caucasians, such that there are only two common haplotypes defined by the presence or absence of the *Taq*I restriction site (22–24). It has been suggested that these haplotypes are associated with differences in bone mineral density and risk of osteoporosis (22), however, the evidence for this is controversial (25). The polymorphisms used are considered to be neutral markers and the functional VDR variant has not yet been identified. Polymorphisms of the VDR gene have also been examined in prostate cancer association studies (16,24,26) and there is some evidence that certain genotypes may be associated with altered risk, in particular Caucasians homozygous for the presence of the *Taq*I site may have a reduced risk of prostate cancer (24). Reports on Japanese and African-American populations have also suggested associations between certain VDR genotypes and breast cancer risk (27,28). Here we have investigated this association in the British population.

Materials and methods

Population series

All patients and controls in this study were Caucasian females from the East Anglian region of the UK. Two separate series (Strata) were used to overcome the need to increase thresholds of statistical significance when carrying out multiple tests. The selection criteria for the two series differed slightly and the sets of results were initially examined individually and only combined if found to be similar.

The first series were a prospectively ascertained group of 288 incident patients attending the Addenbrooke's Hospital (East Anglia) for treatment between 1992 and 1995 and diagnosed below age 71 years (mean 52.5 ± 12.8, range 28.6–70.8 years). These were compared with a group of 288 randomly selected, anonymous controls taken from the EPIC study (29), a population-based cohort study of diet and health (mean age 58.9 ± 9.2, range 44.7–75.6 years). This cohort contains ~25 000 individuals resident in Norfolk (East Anglia) recruited from 1992 to 1996.

The second series were a retrospectively ascertained group of patients identified through the East Anglian Cancer Registry, as part of the Anglian Breast Cancer Study, comprising all patients diagnosed below age 55 years since 1991 and still alive in 1996 (mean 46.6 ± 5.7, range 25.0–54.9 years). These were compared with a second group of 384 random controls also from the EPIC cohort (mean age 55.6 ± 8.1, range 39.9–69.9 years).

A 9 ml EDTA whole blood sample was taken from each study subject for DNA extraction. DNA was extracted consecutively as the blood samples arrived in the laboratory. At the time of the AR gene analysis DNA was available from a total of 508 cases and 426 controls. By the time of the analysis of the VDR gene, samples were available from a further 443 cases and 201 controls. DNA samples that did not amplify under standard PCR conditions (given below) were excluded from analysis.

DNA analysis

Androgen receptor. Separate PCR reactions were carried out across the two polymorphic loci on each DNA sample analysed. The primers for the poly[Gly]$_n$ tract were AR13 (***act ctc ttc aca gcc gaa gaa ggc***) and AR16 (***atc agg tgc ggt gaa gtc gct ttc c***). AR16 was labelled with either the fluorescent dye JOE (PE Applied Biosystems, Warrington, UK) for cases or FAM for controls. PCR was carried out using Amplitaq Gold™ (PE Applied Biosystems) according to the manufacturer's recommended conditions and using a primer annealing temperature of 57°C in a buffer containing 3:1 deaxa-GTP:GTP to lower the melting temperature of this GC-rich template. Fragments in the range 156–213 bp were obtained. The primers for the poly[Gln]$_n$ tract were ARa (***acc agg tag cct gtg ggg cct cta cga tgg gc***) and ARb (***cca gag cgt gcg cga agt gat cca aga acc cgg***). ARb was also labelled with either JOE for cases or FAM for controls. PCR was carried out using Amplitaq Gold™ according to the manufacturer's recommended conditions using a primer annealing temperature of 55°C to give fragments in the size range 205–299 bp. A multiplex of both PCR products from a single case sample and both from a single control together with GS-500 ROX size marker and loading buffer (PE Applied Biosystems) was made and loaded into a single lane of Sequagel-6 matrix (National Diagnostics, Hull, UK) and detected on a model 373 sequencer (PE Applied Biosystems). The pairing of case and control individuals in a single lane overcame the discrepancies in sizing across the gels; in addition a homozygous sample of known size was loaded onto each gel to overcome sizing differences between gels. Band sizes were analysed using Genotyper™ software (PE Applied Biosystems).

Vitamin D receptor. A 400 bp PCR across the boundary of intron 8 and exon 9 of the VDR gene was carried out using primers I8F (***cag agc atg gac agg gag caa g***) and E9R (***tgg atc atc ttg gca tag agc agg***) (24) using Red-Hot DNA Polymerase (Advanced Biotechnologies, Epsom, UK) according to the manufacturer's recommended conditions, using a primer annealing temperature of 47°C. PCR products were digested with *Taq*I enzyme (New England Biolabs, Hitchin, UK) to give fragments of 300 + 100 bp in the presence of the polymorphic cutting site. Digested fragments were separated on 4% Nusieve GTG agarose (Flowgen, Lichfield, UK).

Statistical methods

Associations between polymorphisms and breast cancer risk were analysed by logistic regression using the program S-plus. Cases and controls were genotyped in two groups: Stratum A (288 cases and 288 controls) and Stratum B (up to 672 cases and 384 controls) and all analyses allowed for strata as a covariate.

The effects of the AR repeats were first assessed by testing for a trend in breast cancer risk with repeat length by fitting a parameter for repeat length (averaged over the two chromosomes) in logistic regression. We also treated the AR repeats as a di-allelic marker by dividing into long and short repeat lengths using values that have been used in other studies (12,13,19,20).

The linkage disequilibrium parameter (Δ) between the AR polymorphisms was calculated using maximum likelihood haplotype frequencies, using the AR allele length divisions suggested by Irvine *et al.* (30).

Results

Androgen receptor

DNA from series 1 and 2 was genotyped at the poly[Gly]$_n$ and poly[Gln]$_n$ tracts. There are no significant differences between the results obtained for the two different series (data not shown). The combined results (from a total of 508 cases and 426 controls) are shown in Figure 1. The poly[Gly]$_n$ allele distribution has a range of 4–23 repeats with 17 repeats as the most common allele, while the poly[Gln]$_n$ tract approximates to a normal distribution with a range of 10–39 and a mode of 23 repeats. Inspection of the individual allele frequencies reveals no differences between cases and controls greater than would be expected by chance.

The poly[Gln]$_n$ alleles were observed in 129 different genotype classes (data not shown, but are available from the corresponding author on request). There is no trend for longer genotypes of either tract (expressed as mean length of an individual's two alleles) to be associated with either increasing or decreasing risk: for the poly[Gly]$_n$ tract the mean genotype is 13.8 repeats in controls and 14.0 in cases and the OR is 1.07 (95% CI 0.89–1.48) for each additional repeat carried, whilst for the poly[Gln]$_n$ tract the mean genotype is 23.5 repeats in both cases and controls [OR = 0.99 (95% CI 0.93–1.04) per repeat]. Since poly[Gln]$_n$ tract lengths have been shown to be inversely related to AR transactivation efficiencies (10,11), we have specifically looked at the genotypes of this tract and have considered genotypes involving alleles at each end of the poly[Gln]$_n$ range. These results are shown in Table I. Neither heterozygous carriers nor homozygotes for alleles

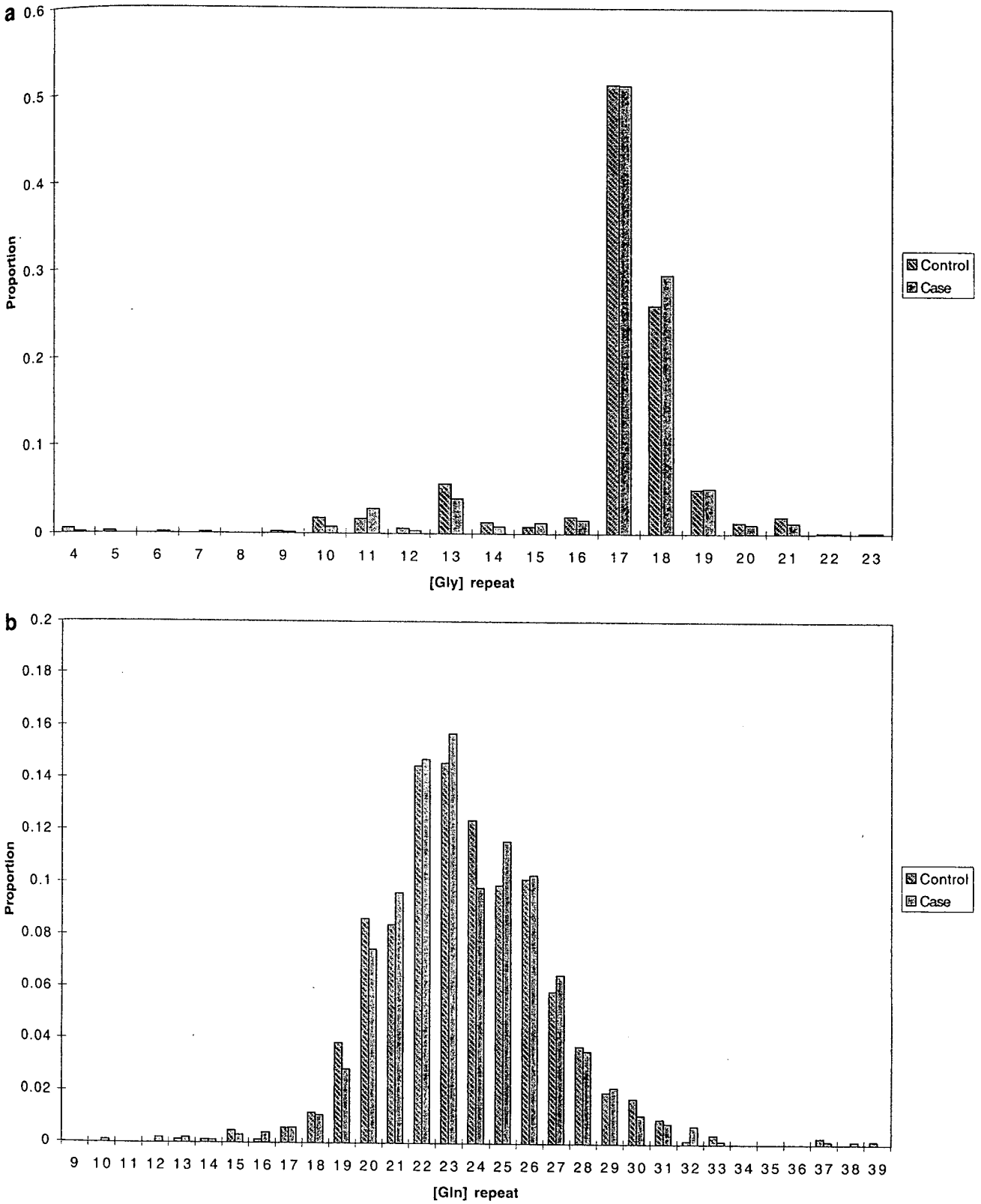

Fig. 1. Androgen receptor allele distributions in breast cancer cases and controls. Allele frequencies for the poly[Gly] tract (**a**) and the poly[Gln] tract (**b**) were determined by gene counting and the relative proportions of each repeat length allele in series 1 and 2 combined are shown.

<22 repeats or >29 repeats show significant differences in breast cancer risk. In series 2 there is some suggestion that the heterozygous carriers of an allele <22 repeats may be protected from breast cancer [OR = 0.67 (95% CI 0.46–0.98)], but this result becomes insignificant when the control genotypes are corrected for Hardy–Weinberg equilibrium.

In contrast to a previous report on a very small sample of males (30), there is no evidence for linkage disequilibrium

Table I. The AR poly[Gln] genotypes with potentially different transactivation efficiencies and breast cancer risk

	ss	sl	ll	OR (sl)	OR (ss)
<22 (s) repeats versus >23 (l) repeats					
Series 1: Addenbrooke's incident patients versus EPIC A controls					
Cases	26	96	112	1.07	1.41
Controls	13	63	79	(0.70–1.65)	(0.68–2.91)
Series 2: ABC prevalent patients versus EPIC B controls					
Cases	58	119	97	0.67	1.18
Controls	41	144	81	(0.46–0.98)	(0.72–1.94)
Combined results					
Cases	84	215	209	0.82	1.31
Controls	54	212	160	(0.62–1.09)	(0.87–1.97)
>29 (l) repeats versus <28 (s) repeats					
Series 1: Addenbrooke's incident patients versus EPIC A controls					
Cases	208	23	3	0.95	0.99
Controls	137	16	2	(0.48–1.86)	(0.16–5.99)
Series 2: ABC prevalent patients versus EPIC B controls					
Cases	254	19	1	0.73	0.96
Controls	245	25	1	(0.39–1.37)	(0.06–15.51)
Combined results					
Cases	462	42	4	0.82	0.97
Controls	382	41	3	(0.52–1.30)	(0.21–4.42)

Table II. The VDR *Taq*I RFLP and breast cancer risk

	TT	Tt	tt	OR (Tt)	OR (tt)
Series 1: Addenbrooke's incident patients versus EPIC A controls					
Cases	85	99	27	1.01	0.79
Controls	105	121	42	(0.68–1.49)	(0.45–1.39)
Series 2: ABC prevalent patients versus EPIC B controls					
Cases	260	354	126	1.01	1.05
Controls	128	172	59	(0.77–1.34)	(0.72–1.53)
Combined results					
Cases	345	453	153	1.01	0.97
Controls	233	293	101	(0.81–1.27)	(0.71–1.32)

between these two loci: $\Delta = -0.09$ in controls and 0.017 in cases and neither of these values reaches statistical significance.

Vitamin D receptor

In total 951 cases and 627 controls were genotyped for the VDR *Taq*I restriction fragment length polymorphism (RFLP) and the genotype distributions are shown in Table II. Again, the results from the two case–control series are very similar. The rarer **t** allele (presence of the *Taq*I cutting site) has a frequency of 0.40 in both cases and controls. We observed no significant differences between cases and controls in any genotype class [OR (**Tt**) = 1.01 (95% CI 0.81–1.270, OR (**tt**) = 0.97 (95% CI 0.71–1.32)].

Discussion

From these large, population-based case–control studies we see no evidence that common alleles of either the AR or VDR loci have an effect on risk of breast cancer in the general East Anglian, British population.

Our findings on the poly[Gln]$_n$ tract in the AR gene are in agreement with those of Spurdle *et al.* (31). We find no evidence for female carriers of tracts with >28 Gln to have an increased risk of breast cancer. This became a plausible hypothesis in the light of two other findings: first, that female carriers of such long repeats had an earlier onset of breast cancer if they were also BRCA1 mutation carriers (19); second, that male carriers are also predisposed to breast cancer (20). Both these reports indicated that longer repeats, demonstrated to have reduced transactivation efficiency (11) may confer an increased risk of breast cancer amongst particular subsets of high risk of individuals, but we see no similar effect on general breast cancer risk. Several reports have additionally suggested that short AR glutamine repeats, with increased transactivation efficiency, are associated with a mildly increased risk of prostate cancer (12–16). Again, we see no similar effect on female breast cancer risk [OR (**sl**) = 0.82 (95% CI 0.62–1.09), OR (**ss**) = 1.31 (95% CI 0.87–1.97)].

We also found no effect of the VDR *Taq*I RFLP on breast cancer risk in our population sample. We can exclude substantial effects of this locus since the 95% upper confidence limit for relative risk is 1.32 and the corresponding upper confidence limit for population attributable risk (PAR) is 0.17 [PAR = 0.029 (95% CI 0–0.17)]. This is in contrast to the previously suggested association with the *Bsm*I RFLP in the Japanese population (28): using 60 cases and 120 controls this study reported that the ***bb*** genotype (which is equivalent to the ***TT*** genotype described here; 24) confers an OR for breast cancer of 3.90 (95% CI 1.63–9.30).

The *Taq*I RFLP of the VDR gene has been shown to be in very strong linkage disequilibrium with the other reported VDR polymorphisms in three large series of Caucasians (22–24). It is therefore likely that we can exclude all known VDR haplotypes from having a substantial effect on breast cancer risk in the British population.

We do not have the statistical power, in a study of this size, to carry out meaningful interaction studies, since neither gene shows any major effect on breast cancer risk and the AR gene has so many different genotype classes, and so these have not been attempted here.

We cannot exclude the possibility that different, as yet undescribed, polymorphisms in the VDR or AR are associated with breast cancer risk. However, it seems likely that other genes involved in response to endogenous hormones will be more important than these in determining common breast cancer risk.

Acknowledgements

We would like to thank all the subjects who participated in these studies after giving written, informed consent. Study designs were approved by 10 local ethical committees covering the entire East Anglian region. The authors are also indebted to the EPIC management group and to Victoria Basham, Joanna Dearden, Patricia Harrington, Carol Houghton, Julian Lipscombe, Carole Pye, Karen Redman and Paul Russell for access to, and preparation of, the control and patient DNA samples. This work was funded by grants from the Cancer Research Campaign. B.A.J.P. is a Cancer Research Campaign Gibb Fellow. F.D. is a Hitchings-Elion Fellow of the Burroughs–Wellcome Fund.

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Received May 13, 1999; revised July 5, 1999; accepted July 16, 1999